



UNITED STATES PATENT AND TRADEMARK OFFICE

I, Charles Edward SITCH BA,

Deputy Managing Director of RWS Group Ltd UK Translation Division, of Europa House, Marsham Way, Gerrards Cross, Buckinghamshire, England declare;

1. That I am a citizen of the United Kingdom of Great Britain and Northern Ireland.
2. That the translator responsible for the attached translation is well acquainted with the German and English languages.
3. That the attached is, to the best of RWS Group Ltd knowledge and belief, a true translation into the English language of the accompanying copy of the specification filed with the application for a patent in Germany on 8 June 1999 under the number 199 26 068.0 and the official certificate attached hereto.
4. That I believe that all statements made herein of my own knowledge are true and that all statements made on information and belief are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application in the United States of America or any patent issuing thereon.

For and on behalf of RWS Group Ltd

The 16th day of February 2005

FEDERAL REPUBLIC OF GERMANY

[Eagle crest]

**Priority Certificate
for the filing of a Patent Application**



File Reference: 199 26 068.0

Filing date: 8 June 1999

Applicant/Proprietor: Professor Dr. Arne S k e r r a, Freising/DE

Title: Muteins of the bilin-binding protein

IPC: C 07 K, C 12 N and C 12 Q

The attached documents are a correct and accurate reproduction of the original submission for this Application.

Munich, 7 August 2000

German Patent and Trademark Office

The President

pp

[Seal of the German Patent
and Trademark Office].

[signature]

Seiler

19 **FEDERAL REPUBLIC
OF GERMANY**

[crest]

**GERMAN PATENT
OFFICE**

12 **Patent Specification**
11 **DE 199 26 068 C 1**

51 Int. Cl.⁷:
C 07 K 14/435
C 12 N 15/62
C 12 Q 1/68

21 File reference 199 26 068.0-41
22 Date of application 8. 6. 1999
43 Date laid open -
46 Date of publication of 11. 1.2001
the grant of the patent:

Opposition can be notified within 3 months from the publication of grant

73 Patent proprietor:
Skerra, Arne, Prof. D0072., 85354 Freising, DE

72 Inventors:
Skerra, Arne, Prof. Dr., 85354 Freising, DE;
Schlehuber, Steffen, Dipl.-Ing., 85356 Freising,
DE

56 Printed publications taken into consideration for
assessing patentability:
Eur. J. Biochem. 219, S. 855-863, 1994;

54 (54) Muteine des Bilin-Bindungsproteins

(57) Die Erfindung bezieht sich auf Muteine des Bilin-Bin-
dungsproteins mit Bindungsfähigkeit für Digoxigenin so-
wie Fusionsproteine solcher Muteine, Verfahren zur Her-
stellung derartiger Muteine und ihrer Fusionsproteine so-
wie deren Verwendung zum Nachweis oder zur Bindung
von mit Digoxigenin markierten Biomolekülen. Insbeson-
dere betrifft die Erfindung ein Polypeptid, ausgewählt aus
Muteinen des Bilin-Bindungsproteins, welches dadurch
gekennzeichnet ist, daß es (a) Digoxigenin oder Konjuga-
te des Digoxigenins zu binden vermag, (b) Ouabain, Te-
stosteron und 4-Aminofluorescein nicht bindet und (c) an
mindestens einer der Sequenzpositionen 28, 31, 34, 35,
36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125 und 127 des
Bilin-Bindungsproteins eine Aminosäuresubstitution auf-
weist. Aufgrund ihres einfachen molekularen Aufbaus
weisen die erfindungsgemäßen Muteine bei Herstellung
und Verwendung Vorteile im Vergleich zu Antikörpern ge-
gen die Digoxigeningruppe auf.

BUNDESDRUCKEREI 11.00 002 162/146/7A

DE 199 26 068 C 1



Description

5 The present invention relates to muteins of the bilin-binding protein which are capable of binding digoxigenin and to fusion proteins of such muteins, to methods for preparing muteins of this kind and their fusion proteins and also to the use thereof for
10 detecting or binding biomolecules labeled with digoxigenin.

In molecular biology, the digoxigenin group is these days a very common instrument for nonradioactive
15 detection of nucleic acids, proteins and other biomolecules. For this purpose, the biomolecule is, mostly covalently, modified with a reactive digoxigenin derivative, thus allowing subsequent detection of the molecule using an antibody directed against the
20 digoxigenin group or a conjugate of an appropriate antibody fragment and a reporter enzyme, according to generally used methods in biochemistry.

The skilled worker knows quite a number of reactive
25 digoxigenin derivatives which are partially also commercially available. For example, digoxigenin-3-O-methylcarbonyl- ϵ -aminocaproic acid N-hydroxysuccinimide ester (DIG-NHS), digoxigenin-3-O-succinyl- ϵ -aminocaproic acid N-hydroxysuccinimide ester and 3-amino-3-
30 deoxydigoxigenin-hemisuccinimide succinimidyl ester are suitable for covalent coupling to proteins, in particular to the amino groups of exposed lysine side chains. Using 3-iodoacetyl-amino-3-deoxydigoxigenin it is possible to label especially thiol groups in
35 proteins or in other biomolecules selectively with the digoxigenin group. It is possible to couple synthetic oligodeoxynucleotides to the same reactive digoxigenin derivatives, as long as they have been provided with suitable free amino or thiol groups during synthesis.

In addition, cis-platinum complexes of digoxigenin derivatives (DIG Chem-Link reagent) or digoxigenin derivatives containing carbodiimide groups (disclosed in the European patent specification EP 0 806 431 A2) are suitable for direct labeling of nucleic acids. Alternatively, it is possible in the case of deoxyribonucleic acids to label said deoxyribonucleic acids during a matrix-dependent enzymic synthesis with the aid of a DNA polymerase and a deoxynucleotide triphosphate coupled to the digoxigenin group, for example digoxigenin-11-dUTP, digoxigenin-11-ddUTP or digoxigenin-16-dATP. Analogously, digoxigenin-11-UTP is suitable for incorporation into enzymically synthesized RNA. Moreover, it is possible to label oligodeoxynucleotides with the digoxigenin group directly in the automated DNA synthesis by using suitable activated building blocks, for example "virtual nucleotides". Digoxigenin group-coupled nucleic acids of this kind are suitable as nonradioactive gene probes for detection of complementary nucleotide sequences by hybridization, for example in Northern or Southern blots (disclosed in the European patent specification EP 0 324 474 A1).

Digoxigenin group-labeled proteins or glycoproteins are particularly useful for determining, for example, relevant antigens or antibodies directed thereagainst in immunochemical assay methods such as ELISA (enzyme-linked immunosorbent assay). The biomolecule conjugated with the digoxigenin group is actually detected using an anti-digoxigenin antibody, normally in the form of a conjugate of the Fab fragment of said antibody with a suitable enzyme, such as, for example, alkaline phosphatase or horseradish peroxidase, as label. The enzymic activity then serves for quantification via catalysis of a chromogenic, fluorogenic or chemiluminescent reaction. Various antibodies against the digoxigenin group are known (Mudgett-Hunter et al.,

J. Immunol. 129 (1982), 1165-1172; Jeffrey et al., J. Mol. Biol. 248 (1995), 344-360).

The use of antibodies, however, has several
5 disadvantages. Thus, preparation of monoclonal
antibodies in hybridoma cell cultures is complicated,
and proteolysis to give the Fab fragment and also
production of conjugates with reporter enzymes requires
additional difficult process steps. But even the
10 production of antibodies by genetic engineering is not
simple, and the main reason for this is that antibodies
as well as antigen-binding fragments thereof are
composed of two different polypeptide chains in a
structurally complicated manner. For genetic
15 manipulation of antibodies it is therefore necessary to
handle two genes simultaneously. Moreover, the yield of
correctly folded antibody fragments produced by genetic
engineering is often low. As is known to the skilled
worker, this is even more so when recombinant fusion
20 proteins are to be prepared from Fab fragments of
antibodies and enzymes.

It was therefore the object of the invention to develop
alternative polypeptide reagents for detection of the
25 digoxigenin group, which can be produced in a simple
manner.

In an evolutive research approach, it has surprisingly
been found now that muteins of the bilin-binding
30 protein which is structurally based on a single
polypeptide chain (Schmidt and Skerra, Eur. J. Biochem.
219 (1994), 855-863) are suitable for detecting the
digoxigenin group by high-affinity binding, with
digoxigenin recognition being astoundingly selective
35 compared with other steroids.

The present invention thus relates to a polypeptide,
selected from muteins of the bilin-binding protein,
which is characterized in that it

(a) is able to bind digoxigenin or digoxigenin conjugates,

(b) does not bind ouabain, testosterone and 4-aminofluorescein and

5 (c) has an amino acid substitution at at least one of the sequence positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125 and 127 of the bilin-binding protein.

10 Outside the region of amino acid positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125 and 127 the muteins of the present invention may correspond to the amino acid sequence of the bilin-binding protein from *Pieris brassicae*. On the other
15 hand, the amino acid sequence of the polypeptides of the invention may have differences to the bilin-binding protein also besides said positions. Bilin-binding protein sequence variants of this kind comprise naturally occurring and also artificially generated
20 variants, and the deviations mean substitutions, insertions, deletions of amino acid residues and also N- and/or C-terminal additions.

For example, the inventive muteins of the bilin-binding
25 protein may have amino acid substitutions which prevent oligomerization of the bilin-binding protein, such as the Asn(1)->Asp substitution, or suppress proteolytic cleavage within the polypeptide chain, which may occur during production in *E. coli*, such as, for example, the
30 Lys(87)->Ser substitution. Furthermore, it is possible to introduce the mutations Asn(21)->Gln and Lys(135)->Met into the nucleic acids coding for the muteins of the bilin-binding protein, in order to facilitate, for example, cloning of a gene segment via
35 two new *Bst*XI restriction cleavage sites at these positions. Likewise, the present invention relates to the specific introduction of amino acid substitutions within or outside the said positions, in order to generally improve particular properties of the mutein

of the invention, for example its folding stability or folding efficiency or its resistance to proteases.

5 The ability of the polypeptides of the invention to bind digoxigenin or digoxigenin conjugates can be determined by common methods, for example ELISA, fluorescence titration, titration calorimetry, surface plasmon resonance measurements or blotting methods, for example Western blotting, Southern blotting or Northern
10 blotting. Blotting methods may be used in order to transfer conjugates of digoxigenin with proteins or nucleic acids to a membrane and then detect said conjugates using one of the muteins of the invention, a conjugate of this mutein or a fusion protein of this
15 mutein.

A quantitative parameter for binding affinity is provided by established thermodynamic parameters such as, for example, the affinity constant or dissociation
20 constant for the complex of mutein and bound ligand, for example digoxigenin. However, it is also possible to determine the binding ability qualitatively, for example based on the intensity of a binding signal due to a chromogenic reaction or of a colored precipitate
25 which is obtained with the aid of one of said blotting methods.

Preferred muteins of the invention are obtainable in a two-stage evolutive process. Random mutagenesis of the
30 bilin-binding protein and repeated selection of muteins with digoxigenin group affinity from this library, using free digoxigenin for competitive concentration, provides muteins of the bilin-binding protein which recognize the digoxigenin group, but the affinity is
35 still comparatively low. Renewed mutagenesis of such a mutein at amino acid positions 28, 31, 34, 35, 36 and 37, now followed by a repeated concentration by formation of a complex with the digoxigenin group and by subsequent dissociation of the complex formed in

acidic medium, then results in obtaining muteins having substantially higher affinity for the digoxigenin group.

5 Surprisingly, it has now been found that the affinity constant between such polypeptides of the invention and digoxigenin is at least 10^7 M^{-1} . This means in other words that the dissociation constant of the complex of the polypeptide of the invention and digoxigenin is
10 100 nM or less. Individual examples even show dissociation constants of 35 nM or less, as illustrated in the examples.

Besides digoxigenin, the inventive muteins of the
15 bilin-binding protein can also bind digoxigenin derivatives as ligands, for example digoxin, digitoxin or digitoxigenin. Furthermore, the inventive muteins of the bilin-binding protein may bind conjugates of said chemical compounds, i.e. nucleic acids, polypeptides,
20 carbohydrates, other natural or synthetic biomolecules, macromolecules or low molecular weight compounds which are covalently linked or linked via a metal complex to digoxigenin, digoxin, digitoxin or digitoxigenin. Preference is given to using for the preparation of
25 such conjugates the reactive derivatives of digoxigenin, digoxin, digitoxin or digitoxigenin, which are known to the skilled worker and are stated, for example, further above.

30 Preferred muteins of the invention, which were obtained by the two-stage process described, show, compared with the affinity for digoxigenin, an even higher affinity for digitoxin or digitoxigenin whose steroid system differs from that of digoxigenin only by the absence of
35 a hydroxyl group. Surprisingly, these muteins show distinctive specificity with respect to the digoxigenin or digitoxigenin group, and this is shown by the fact that other steroids or steroid groups such as ouabain or testosterone are bound with much less affinity, if

at all. Fluorescein derivatives such as 4-amino-fluorescein, too, are evidently not bound. This means that ouabain, testosterone or 4-aminofluorescein in each case have a dissociation constant of at least
5 10 μ M, preferably at least 100 μ M for the inventive muteins of the bilin-binding protein.

This property of specificity distinguishes said muteins considerably from other muteins of the bilin-binding
10 protein and also from antibodies directed against the digoxigenin group, such as, for example, antibody 26-10 (Chen et al., Protein Eng. 12 (1999), 349-356) which binds ouabain with substantial affinity, and gives the inventive muteins of the bilin-binding protein a
15 particular advantage. It is surprising that particularly the additional amino acid substitutions at positions 28, 31, 34, 35, 36 and 37 lead to the preferred muteins of the bilin-binding protein. Preference is therefore given to those muteins which
20 carry at least one or all of the amino acid substitutions Glu(28)->Gln, Lys(31)->Ala, Asn(34)->Asp, Ser(35)->His, Val(36)->Ile and Glu(37)->Thr.

Particularly preferred muteins of the invention carry,
25 when compared to the bilin-binding protein, at least one of the amino acid substitutions selected from Glu(28)->Gln, Lys(31)->Ala, Asn(34)->Asp, Ser(35)->His, Val(36)->Ile, Glu(37)->Thr, Asn(58)->Arg, His(60)->Ser, Ile(69)->Ser, Leu(88)->Tyr, Tyr(90)->Ile, Lys(95)->Gln,
30 Asn(97)->Gly, Tyr(114)->Phe, Lys(116)->Ser, Gln(125)->Met and Phe(127)->Leu. The representation chosen indicates in each case first the amino acid in the natural bilin-binding protein (SWISS-PROT database accession code P09464) together with the sequence
35 position for the mature polypeptide in brackets, and the corresponding amino acid in a mutein of the invention is stated after the arrow. Very particularly preferred muteins according to this invention carry all of the amino acid substitutions mentioned.

Surprisingly, bilin-binding protein position 93 is unchanged in the muteins of the invention, although this amino acid, too, had been affected by the mutagenesis for preparing the random library. Preferred
5 muteins of the bilin-binding protein therefore carry the amino acid Val at said position.

It is an advantage for particular detection methods to use the muteins of the bilin-binding protein of the
10 present invention in a labeled form. Accordingly, this invention further relates to a polypeptide of the invention, which is characterized in that it carries at least one label. Suitable labeling groups are known to the skilled worker and include enzymic label,
15 radioactive label, fluorescent label, chromophoric label, (bio)luminescent label or a label containing haptens, biotin, metal complexes, metals or colloidal gold. Very generally, labeling is possible with substances or enzymes which generate a determinable
20 substance in a chemical or enzymatic reaction. In this connection it is possible to couple all known labels for antibodies to the muteins of the invention, too.

A possibility which is particularly advantageous for
25 practical application is to use the inventive muteins of the bilin-binding protein in the form of fusion proteins. Techniques for preparing such fusion proteins by means of genetic engineering methods are known to the skilled worker. Suitable fusion partners for the
30 muteins of the invention would be enzymes and other polypeptides, proteins or protein domains. Such fusions would be suitable for providing the mutein of the bilin-binding protein with additional properties such as, for example, enzymic activity or affinity for other
35 molecules such as proteins, macromolecules or low molecular weight ligands.

Examples of possible fusions are those with enzymes which catalyze chromogenic or fluorogenic reactions or

may be used for the release of cytotoxic agents. Further examples for fusion partners which may be advantageous in practice are binding domains such as the albumin-binding domain or the immunoglobulin-binding domain of protein G or protein A, antibody fragments, oligomerization domains, toxins or other binding proteins and functional parts thereof and also affinity peptides such as, for example, Strep tag or Strep tag II (Schmidt et al., J. Mol. Biol. 255 (1996), 753-766). Suitable fusion partners are also proteins having particular chromogenic or fluorogenic properties, such as, for example, green fluorescent protein. Another suitable fusion partner would be coat protein III of a filamentous bacteriophage such as M13, f1 or fd, or a fragment of said coat protein.

Very generally, the term fusion protein is intended here to mean also those inventive muteins of the bilin-binding protein, which are equipped with a signal sequence. Signal sequences on the N terminus of the polypeptide of the invention may serve the purpose of directing said polypeptide during biosynthesis into a particular cell compartment, for example the *E. coli* periplasm or the lumen of the endoplasmic reticulum of a eukaryotic cell, or into the medium surrounding the cell. The signal sequence is typically cleaved off in the process by a signal peptidase. In addition, it is also possible to use other signal or targeting sequences which need not necessarily be located on the N terminus of the polypeptide and which make it possible to locate said polypeptide in specific cell compartments. A preferred signal sequence for secretion into the *E. coli* periplasm is the ompA signal sequence. A large number of further signal sequences and also targeting sequences are known in the prior art.

An advantage of the inventive muteins of the bilin-binding protein is the suitability of both their N terminus and their C terminus for preparing fusion

proteins. In contrast to antibodies in which the N terminus of both the light and the heavy immunoglobulin chain is in spatial proximity to the antigen binding site, it is possible to use in the polypeptides of the invention both ends of the polypeptide chain for the preparation of fusion proteins, without adversely affecting ligand binding.

The invention therefore also relates to fusion proteins of muteins of the bilin-binding protein, in which an enzyme, another protein or a protein domain, a signal sequence and/or an affinity peptide is fused to the amino terminus of the polypeptide in an operable manner. The invention yet further relates to fusion proteins of bilin-binding protein muteins or of fusion proteins having the amino terminus of bilin-binding protein muteins, in which an enzyme, another protein or a protein domain, a targeting sequence and/or an affinity peptide is fused to the carboxy terminus of the polypeptide in an operable manner.

A preferred enzyme for constructing the fusion proteins of the invention is bacterial alkaline phosphatase (Sowadski et al., J. Mol. Biol. 186 (1985) 417-433) which may be attached either at the N terminus or at the C terminus of a mutein of the bilin-binding protein. In addition, such a fusion protein may carry a signal sequence such as, for example, OmPA or PhoA, which effect secretion of said fusion protein into the *E. coli* periplasm where the disulfide bonds may form efficiently in the polypeptide chain. Furthermore, it may be equipped with an affinity peptide such as, for example, Strep tag II, which allows easy purification of said fusion protein. The specific fusion proteins of the invention are described in the examples. An advantage of a fusion protein of this kind is its ability to catalyze directly a chromogenic, fluorogenic or chemiluminescent detection reaction, which

simplifies its use for detection of the digoxigenin group.

Another advantage of using alkaline phosphatase for
5 constructing fusion proteins of the invention is the
fact that this enzyme forms a stable homodimer and,
consequently, confers the property of bivalence on the
bilin-binding protein mutein as part of the fusion
protein. In this way, binding of the digoxigenin group
10 may result in avidity effect which increases detection
sensitivity. Such an avidity effect can be expected in
particular if the digoxigenin-labeled molecule is
adsorbed to a solid phase, is present in oligomeric or
membrane-bound form or is conjugated with a plurality
15 of digoxigenin groups. Analogously, other homodimeric
enzymes are suitable for preparing bivalent fusion
proteins containing the inventive muteins of the bilin-
binding protein.

20 Apart from bacterial alkaline phosphatase, it is also
possible to use phosphatases from eukaryotic organisms,
such as, for example, calf intestine phosphatase (CIP),
for preparing fusion proteins of the invention. Said
phosphatases are frequently distinguished by higher
25 enzymatic activity (Murphy and Kantrowitz, Mol.
Microbiol. 12 (1994), 351-357), which may result in
higher detection sensitivity. It is also possible to
use mutants of bacterial alkaline phosphatase, which
have improved catalytic activity (Mandecki et al.,
30 Protein Eng. 4 (1991), 801-804), for constructing
fusion proteins of the invention. Other enzymes known
to the skilled worker which catalyze chromogenic,
fluorogenic or chemiluminescent reactions, such as, for
example, β -galactosidase or horseradish peroxidase, are
35 also suitable for preparing fusion proteins of the
invention. Moreover, all these enzymes may likewise be
employed for labeling muteins of the bilin-binding
protein by conjugating them, for example by using

common coupling reagents, with the mutein obtained separately or a fusion protein of the mutein.

5 In another aspect, the present invention relates to a nucleic acid which comprises a sequence coding for a mutein or a fusion protein of a mutein of the bilin-binding protein. This nucleic acid may be part of a vector which contains operatively functional areas for expressing the nucleic acid. A large number of suitable
10 vectors is known from the prior art and is not described in detail here. Operatively functional areas are those elements which allow, assist, facilitate and/or increase transcription and/or subsequent processing of an mRNA. Examples of elements of this
15 kind include promoters, enhancers, transcription initiation sites and transcription termination sites, translation initiation sites, polyadenylation signals, etc.

20 The nucleic acid of the invention or its surrounding areas may be such that biosynthesis of the polypeptide takes place in the cytosol, the polypeptide sequence being preceded, where appropriate, by a starting methionine. In a preferred embodiment, however, an
25 N-terminal signal sequence is used, in particular the OmpA or PhoA signal sequence, in order to direct the polypeptide of the invention into the *E. coli* periplasm where the signal sequence is cleaved off by the signal peptidase and the polypeptide chain is able to fold
30 with oxidative formation of the disulfide bonds. Eukaryotic signal sequences may be used in order to secrete the polypeptide of the invention in a eukaryotic host organism. In principle, both prokaryotic, preferably *E. coli*, and eukaryotic cells
35 such as, for example, yeasts are considered for expression of the nucleic acid of the invention.

In yet another aspect, the present invention relates to a method for preparing an inventive mutein or fusion

protein of a mutein of the bilin-binding protein, which method is characterized in that the nucleic acid coding for the mutein or the fusion protein of a mutein of the bilin-binding protein is expressed in a bacterial or eukaryotic host cell and the polypeptide is obtained from the cell or the culture supernatant. For this purpose, normally a suitable host cell is first transformed with a vector which comprises a nucleic acid coding for a polypeptide of the invention. The host cell is then cultured under conditions under which the polypeptide is biosynthesized, and the polypeptide of the invention is obtained.

With respect to the preparation method, it must be taken into account that the inventive muteins of the bilin-binding protein have two structural disulfide bonds and that additional disulfide bonds may be present in corresponding fusion proteins. The formation of said disulfide bonds, which takes place during protein folding, is normally ensured if the polypeptide of the invention is directed with the aid of a suitable signal sequence into a cell compartment containing an oxidizing thiol/disulfide redox medium, for example into the bacterial periplasm or the lumen of the endoplasmic reticulum of a eukaryotic cell. In this connection, the polypeptide of the invention can be liberated by cell fractionation or obtained from the culture supernatant. It is possible, where appropriate, to increase the folding efficiency by overproducing protein disulfide isomerases, for example *E. coli* DsbC protein, or auxiliary folding proteins.

On the other hand, it is possible to produce a polypeptide of the invention in the cytosol of a host cell, preferably *E. coli*. The said polypeptide may then be obtained, for example, in the form of inclusion bodies and then be renatured *in vitro*. Depending on the intended use, the protein can be purified by means of various methods known to the skilled worker. A suitable

method for purifying the inventive muteins of the bilin-binding protein is, for example, affinity chromatography using a column material which carries digoxigenin groups. In order to purify fusion proteins
5 of the muteins of the bilin-binding protein, it is possible to utilize the affinity properties of the fusion protein, which are known from the prior art, for example those of the Strep tag or the Strep tag II (Schmidt and Skerra, J. Chromatogr. A 676 (1994), 337-
10 345; Voss and Skerra, Protein Eng. 10 (1997), 975-982), those of the albumin binding domain (Nygren et al., J. Mol. Recogn. 1 (1988), 69-74) or those of alkaline phosphatase (McCafferty et al., Protein Eng. 4 (1991) 955-961). The fact that the muteins of the bilin-
15 binding protein consist only of a single polypeptide chain is advantageous for the methods for preparing the polypeptides of the invention, since no care needs to be taken either of the need for synthesizing a plurality of various polypeptide chains within a cell
20 simultaneously or of different polypeptide chains associating with one another in a functional manner.

The practical application possibilities for the inventive muteins of the bilin-binding protein
25 essentially correspond to those for conventional antibodies or antibody fragments with binding affinity for digoxigenin. Accordingly, the invention also relates to the use of a mutein of the invention or of a fusion protein of a mutein of the bilin-binding protein
30 in a method for detecting, determining, immobilizing or removing digoxigenin or conjugates of digoxigenin with proteins, nucleic acids, carbohydrates, other biological or synthetic macromolecules or low molecular weight chemical compounds.

35

The inventive muteins of the bilin-binding protein or their fusion proteins can be used in detection methods essentially in a manner analogous to corresponding detection methods known for anti-digoxigenin antibodies

and also fragments and so-called conjugates thereof. In a further aspect, the present invention therefore relates to a method for detecting the digoxigenin group, in which method a mutein of the bilin-binding protein or a fusion protein of a mutein of the bilin-binding protein is contacted with digoxigenin or with digoxigenin conjugates under conditions suitable for effecting binding of the mutein to the digoxigenin group and the mutein or the fusion protein of the mutein is determined.

For this purpose, the mutein may be labeled directly, for example by covalent coupling. It is, however, also possible to use indirect labeling, for example by means of labeled antibodies against the bilin-binding protein or muteins thereof or against domains of fusion proteins of these muteins. The use of inventive fusion proteins containing an enzyme, for example alkaline phosphatase, instead of a labeled mutein of the bilin-binding protein is particularly advantageous. In this case, it is possible to design the determination method with a particularly small number of process steps, it being possible to utilize directly, for example, the ability of the enzyme as part of the fusion protein to catalyze a chromogenic, fluorogenic or luminescent detection reaction. Here, the fact that such fusion proteins are readily available is a particular advantage compared with corresponding fusion proteins of conventional antibodies. Utilization of the abovedescribed avidity effect in the case of an oligomeric fusion protein is another advantage in such a method.

It is possible to carry out a method for determining the digoxigenin group, for example, qualitatively for detecting nucleic acids conjugated with the digoxigenin group in Southern or Northern blots or proteins conjugated with the digoxigenin group in Western blots. A determination method may also be carried out

quantitatively for detecting proteins conjugated with the digoxigenin group in an ELISA. In addition, a determination method of the invention is also suitable for indirect detection of proteins not conjugated with digoxigenin or of other molecules by using a binding protein which is directed against the protein or molecule, for example an antibody or its fragment, and which is conjugated with the digoxigenin group. Indirect detection of the nucleic acids not conjugated with digoxigenin is also possible by using a gene probe which hybridizes with said nucleic acid and which is conjugated with the digoxigenin group. An application in medical diagnostics or therapy results in addition from determination of digoxigenin, digoxin, digitoxin or digitoxigenin, without these ligands having to be conjugated with another molecule.

The muteins of the invention or fusion proteins thereof may also be used for immobilizing a molecule conjugated with the digoxigenin group. This immobilization is preferably carried out on solid phases coated with the muteins or their fusion proteins, such as, for example, microtiter plates, immunosticks, microbeads made of organic, inorganic or paramagnetic materials, or sensor surfaces.

Correspondingly, it is likewise possible to use the muteins of the invention or fusion proteins thereof for removing digoxigenin, digoxin, digitoxin or digitoxigenin, or a molecule conjugated with one of these compounds. In this case, in addition to the solid phases mentioned, column materials are also considered for coating with the muteins or their fusion proteins. Preferably, said coating is carried out on suitable column materials by coupling by means of chemically reactive groups. Column materials coated in this way may be used for removing substances conjugated with digoxigenin groups and also, where appropriate,

complexes of such substances with other molecules from a solution.

Thus, it is possible, for example, to remove antigens
5 from a solution by adding antibodies to the solution, which are directed against the antigens and conjugated with the digoxigenin group, and contacting the resulting solution with said column material under conditions under which a complex between the
10 digoxigenin groups and an inventive mutein of the bilin-binding protein or its fusion protein is formed. Following such a removal, it is also possible, where appropriate, to elute the substance conjugated with digoxigenin. This elution may be carried out by
15 competition with digoxin, digoxigenin, digitoxin or digitoxigenin and also, for example, by lowering or increasing the pH of the solution. In a competitive elution it is possible to utilize in an advantageous manner the higher binding affinity of the muteins of
20 the invention for digitoxigenin or digitoxin compared with the digoxigenin group. In this way it is possible to isolate or purify a substance conjugated with digoxigenin.

25 The invention is further illustrated by the following examples and attached drawings, in which:

Fig. 1 represents in each case a fluorescent titration
of the Strep tag II-fused mutein DigA16 with
30 the ligands digoxigenin, digitoxigenin and ouabain;

Fig. 2 depicts diagrammatically the expression vectors
pBBP27 (A) and pBBP29 (B) for preparing
35 fusion proteins of mutein DigA16 with alkaline phosphatase;

Fig. 3 demonstrates quantitative detection of
biomolecules conjugated with digoxigenin

groups by fusion proteins of mutein DigA16 with alkaline phosphatase in an ELISA;

5 **Fig. 4** shows qualitative detection of biomolecules conjugated with digoxigenin groups by fusion proteins of mutein DigA16 with alkaline phosphatase on a Western blot.

10 **Fig. 1** shows the graphic representation of results from Example 3 in which different concentrations of the steroids digoxigenin (squares), digitoxigenin (circles) and ouabain (rhomboids) were added to a 1 μ M solution of mutein DigA16. The particular protein fluorescence intensities were measured at an excitation wavelength of 295 nm and an emission wavelength of 345 nm and plotted as a function of the actual total steroid concentration in the particular reaction mixture. Finally, the data points were fitted to a regression curve by means of nonlinear regression.

20 **Fig. 2** shows a drawing of the expression vectors pBBP27 (A) and pBBP29 (B). pBBP27 codes for a fusion protein of bacterial alkaline phosphatase with its own signal sequence, a peptide linker having the sequence Pro-Pro-Ser-Ala, the mutein DigA16 and also the Strep tag II affinity tag. The corresponding structural gene is followed by the *dsbC* structural gene (including its ribosomal binding site) from *E. coli* (Zapun et al., Biochemistry 34 (1995), 5075-5089) as second cistron.

30 The artificial operon formed in this way is under joint transcriptional control of the tetracyclin promoter/operator ($tet^{P/o}$) and ends at the lipoprotein transcription terminator (t_{1pp}). Further vector elements are the origin of replication (*ori*), the intergenic region of filamentous bacteriophage f1 (f1-IG), the ampicillin resistance gene (*bla*) coding for β -lactamase and the tetracyclin repressor gene (*tetR*). pBBP29 codes for a fusion protein of the OmpA signal sequence, the mutein DigA16, the Strep tag II affinity tag, a peptide

35

linker consisting of five glycine residues, and bacterial alkaline phosphatase without its N-terminal amino acid arginine. The vector elements outside this region are identical to vector pBBP27.

5

Fig. 3 shows a graphic representation of the data from Example 4 in which digoxigenin groups were detected quantitatively with the aid of mutein DigA16 fusion proteins as gene products of vectors pBBP27 (closed symbols) and pBBP29 (open symbols). Here, the digoxigenin groups were coupled on the one hand to bovine serum albumin (BSA, squares) or, on the other hand, to egg albumin (ovalbumin, triangles). The control data shown are those obtained when using underivatized bovine serum albumin and the fusion protein encoded by pBBP27 (open circles). The enzymic activity corresponding to the particular bound fusion protein was monitored spectrophotometrically at 405 nm on the basis of p-nitrophenyl phosphate hydrolysis. Curve fitting was carried out by nonlinear regression with the aid of the Kaleidagraph computer program (Abelbeck Software) by means of the equation

$$[P \cdot L] = [L]_t [P]_t / (K_d + [P]_t).$$

25

Here, $[P]_t$ corresponds to the total fusion protein concentration used in the particular microtiter plate well. $[P \cdot L]$ is determined on the basis of the enzymic activity of alkaline phosphatase. The total concentration of digoxigenin groups $[L]_t$, constant within a concentration series, per well and the dissociation constant K_d were fitted as parameters by nonlinear regression.

Fig. 4 shows the result of a Western blot experiment from Example 4 for qualitative detection of biomolecules conjugated with digoxigenin groups by means of the mutein DigA16 fusion proteins encoded by pBBP27 (lanes 1 and 2) and pBBP29 (lanes 3 and 4). For

35

comparison, a 15% strength SDS polyacrylamide gel of the biomolecules, stained with Coomassie Brilliant Blue, is likewise shown (lanes 5 and 6). Here, a mixture of 0.5 µg of underivatized BSA, underivatized ovalbumin and underivatized RNaseA was fractionated in each case in lanes 1, 3 and 5. A mixture of 0.5 µg of BSA coupled to digoxigenin groups, ovalbumin coupled to digoxigenin groups and RNaseA coupled to digoxigenin groups was fractionated in each case in lanes 2, 4 and 6.

Examples

Unless stated otherwise, the genetic engineering methods familiar to the skilled worker, as described, for example, in Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989), Cold Spring Harbor Press) were used.

Example 1

Preparation of a library for muteins of the bilin-binding protein, phagemid presentation and selection of a mutein with binding affinity for digoxigenin

A library for muteins of the bilin-binding protein was prepared by subjecting the amino acid sequence positions of said bilin-binding protein, 34, 35, 36, 37, 58, 60, 69, 88, 90, 93, 95, 97, 114, 116, 125 and 127, to a concerted mutagenesis in multiple steps with the aid of the polymerase chain reaction (PCR). The PCR reactions were initially carried out in two separate amplification steps in a volume of in each case 50 µl, and 10 ng of pBBP20 phasmid DNA (SEQ ID NO: 1) as matrix and in each case 25 pmol of two primers (SEQ ID NO. 2 and SEQ ID NO. 3 in one mixture and SEQ ID NO. 4 and SEQ ID NO. 5 in a second mixture) which had been synthesized according to the generally known phosphoramidite method were used.

Furthermore, the reaction mixture contained 5 μ l of 10xTaq buffer (100 mM Tris/HCl pH 9.0, 500 mM KCl, 1% v/v Triton X-100), 3 μ l of 25 mM MgCl₂ and 4 μ l of dNTP mix (2.5 mM dATP, dCTP, dGTP, dTTP). After filling up
5 with water, the mixture was overlaid with mineral oil and heated to 94°C in a programmable thermostating block for 2 min. Then 2.5 u of Taq DNA polymerase (5 u/ μ l, Promega) were added and 20 temperature cycles of 1 min at 94°C, 1 min at 60°C and 1.5 min at 72°C
10 were carried out, followed by an incubation at 60°C for 5 min. The desired amplification products were isolated via preparative agarose gel electrophoresis from low melting point agarose Jetsorb (Gibco BRL), using the DNA extraction kit (Genomed) according to the
15 manufacturer's instructions.

A relevant section of the pBBP20 nucleic acid sequence is shown together with the encoded amino acid sequence as SEQ ID NO. 1 in the sequence listing. The section
20 starts with a hexanucleotide sequence which was obtained by ligating an XbaI overhang with an SpeI overhang complementary thereto and ends with the HindIII cleavage site. The vector elements outside this region are identical to vector pASK75 whoses complete
25 nucleotide sequence is stated in the publication DE 44 17 598 A1.

The subsequent amplification step was carried out in a 100 μ l mixture, and in each case approx. 6 ng of the
30 two isolated fragments as matrix, 50 pmol of each of the two primers SEQ ID NO. 6 and SEQ ID NO. 7 and also 1 pmol of oligodeoxynucleotide SEQ ID NO. 8 were used. The remaining components of the PCR mixture were added in twice the amount, as in the preceding amplification
35 steps. The PCR was carried out in 20 temperature cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C, followed by a final incubation at 60°C for 5 min. The fragment obtained was again isolated by preparative agarose gel electrophoresis.

Said fragment which represented the mutein library in the form of a mixture of nucleic acids was cloned by cutting it first with the restriction enzyme *Bst*XI (New England Biolabs) according to the manufacturer's instructions. The nucleic acid fragment obtained (335 base pairs, bp) was purified again by means of preparative agarose gel electrophoresis. Analogously, pBBP20 vector DNA was cut with *Bst*XI and the larger of the two fragments (3971 bp) was isolated.

10

For ligation, 0.93 µg (4.2 pmol) of the PCR fragment and 11 µg (4.2 pmol) of the vector fragment were incubated in the presence of 102 Weiss units of T4 DNA ligase (New England Biolabs) in a total volume of 500 µl (50 mM Tris/HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 50 µg/ml BSA) at 16°C for two days. The DNA was then precipitated by adding 10 µg of yeast tRNA (Boehringer Mannheim), 25 µl of 5 M ammonium acetate and 100 µl of ethanol to in each case 24 µl of the ligation mixture. Incubation at -20°C for 3 days was followed by centrifugation (25 min, 16000 g, 4°C). The precipitate was washed in each case with 200 µl of ethanol (70% v/v, -20°C) and dried under reduced pressure. Finally, the DNA was taken up in 43.6 µl of TE/10 (1 mM Tris/HCl pH 8.0, 0.1 mM EDTA). The DNA concentration of the solution obtained was estimated by analytical agarose gel electrophoresis on the basis of the fluorescence intensity of the bands stained with ethidium bromide in comparison with a DNA size standard of known concentration.

30

Electrocompetent cells of the *E. coli* K12 strain XL1-Blue (Bullock et al., BioTechniques 5 (1987), 376-379) were prepared according to the methods described by Tung and Chow (Trends Genet. 11 (1995), 128-129) and by Hengen (Trends Biochem. Sci. 21 (1996), 75-76). 1 l of LB medium was adjusted to an optical density at 600 nm, OD₆₀₀ = 0.08 by adding a stationary XL1-Blue overnight culture and incubated in a 3 l Erlenmeyer

35

flask at 200 rpm and 26°C. After reaching $OD_{600} = 0.6$, the culture was cooled on ice for 30 min and then centrifuged at 4000 g and 4°C for 15 min. The cell sediment was washed twice with in each case 500 ml of ice cold 10% w/v glycerol and finally resuspended in 2 ml of ice cold GYT medium (10% w/v glycerol, 0.125% w/v yeast extract, 0.25% w/v tryptone).

Electroporation was carried out by using the Easyjac T Basic system (EquiBio) with the corresponding cuvettes (electrode distance 2 mm). All operational steps were carried out in a cold room at 4°C. 5 to 6 μ l of the above-described DNA solution (245 ng/ μ l) were in each case mixed with 40 μ l of the cell suspension, incubated on ice for 1 min and then transferred into the cuvette. After electroporation, the suspension was immediately diluted in 2 ml of fresh ice-cold SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$) and agitated at 37°C and 200 rpm for 60 min. The cells were then sedimented at 3600 g for in each case 2 min, resuspended in 1 ml of LB medium containing 100 μ g/ml of ampicillin (LB/Amp) and plated out at 200 μ l each on agar plates (140 mm in diameter) with LB/Amp medium. Using a total of 10.7 μ g of the ligated DNA in eight electroporation mixtures produced in this way $3.73 \cdot 10^8$ transformants which were distributed on 40 agar plates.

After incubation at 32°C for 14 h, the colonies obtained in this way were scraped off the agar plates with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension. 50 ml of 2xYT/Amp medium prewarmed to 37°C were inoculated with 2.88 ml of said suspension so that the cell density was 1.0 OD_{550} . This culture was incubated at 37°C, 160 rpm for 6 h to reach a stationary cell density, and phasmid DNA was isolated with the aid of the plasmid Midi kit (Qiagen) according

to the manufacturer's instructions. Finally, the DNA was taken up in 100 µl of TE (10 mM Tris/HCl pH 8.0, 1 mM EDTA) and stored at 4°C for further use.

5 In order to prepare a library of recombinant phagemids (Kay et al., Phage Display of Peptides and Proteins - A Laboratory Manual (1996), Academic Press) which carry the muteins of the bilin-binding protein as a fusion with the truncated coat protein pIII, the phasmid DNA
10 obtained in this way was used for transformation of electrocompetent cells of *E. coli* XL1-Blue. Electroporation was carried out as described above with the aid of the Easyjec T Basic system. In a total of 13 mixtures, 40 µl of the cell suspension of
15 electrocompetent cells were in each case transformed with in each case 2 µg of the DNA in a volume of 5 µl. After electroporation, the cell suspension obtained from each mixture was diluted immediately in 2 ml of fresh ice-cold SOC medium and agitated at 37°C and
20 200 rpm for 60 min.

The mixtures were combined (volume = 26 ml) and 74 ml of 2xYT medium and 100 µl of ampicillin (stock solution 100 mg/ml, final concentration 100 mg/l) were added.
25 The total number of transformants obtained was estimated at $1.1 \cdot 10^{10}$ by plating out 100 µl of a $1:10^5$ dilution of the obtained suspension on agar plates containing LB/Amp medium. After incubation at 37°C and 160 rpm for 60 min, the culture was infected with
30 500 µl of VCS-M13 helper phage ($1.1 \cdot 10^{12}$ pfu/ml, Stratagene) and agitated at 37°C, 160 rpm for a further 60 min. Subsequently, 200 µl of kanamycin (stock solution 35 mg/ml, final concentration 70 mg/l) were added, the incubator temperature was lowered to 26°C
35 and, after 10 min, anhydrotetracyclin (50 µl of a 50 µg/ml stock solution in dimethylformamide, final concentration 25 µg/l) was added to induce gene expression. Finally, the phagemids were produced by incubating the culture at 26°C, 160 rpm for 7 h.

The cells were removed by centrifugation of the culture (15 min, 12000 g, 4°C). The supernatant containing the phagemid particles was sterile-filtered (0.45 µm), mixed with 1/4 volume (25 ml) of 20% w/v PEG 8000, 15%
5 w/v NaCl and incubated at 4°C overnight. After centrifugation (20 min, 18000 g, 4°C), the precipitated phagemid particles were dissolved in a total of 4 ml of cold PBS (4 mM KH₂PO₄, 16 mM Na₂HPO₄, 115 mM NaCl, pH 7.4). The solution was incubated on ice for 30 min and
10 distributed into four 1.5 ml reaction vessels at equal volumes. After removing undissolved components by centrifugation (5 min, 18500 g, 4°C), the supernatant was transferred in each case to a new reaction vessel.

15 The phagemid particles were again precipitated by mixing with 1/4 volume (in each case 0.25 ml per reaction vessel) of 20% w/v PEG 8000, 15% w/v NaCl and incubating on ice for 60 min. After centrifugation (20 min, 18500 g, 4°C), the supernatant was removed and
20 the precipitated phagemid particles were each dissolved in 0.5 ml of PBS. After incubation on ice for 30 min, centrifugation (5 min, 18500 g, 4°C) was repeated to clarify the solution. The supernatant containing the phagemid particles (between $1 \cdot 10^{12}$ and $5 \cdot 10^{12}$ cfu/ml)
25 was then used for affinity concentration.

The recombinant phagemids presenting the muteins of the bilin-binding protein were affinity-concentrated using Immuno Sticks (NUNC). These were coated overnight with
30 800 µl of a conjugate (100 µg/ml) of ribonuclease A (RNaseA) and digoxigenin in PBS.

The conjugate was prepared by adding 1.46 µmol (0.96 mg) of digoxigenin-3-O-methylcarbonyl-ε-amino-caproic acid N-hydroxysuccinimide ester (DIG-NHS,
35 Boehringer Mannheim) in 25 µl of DMSO in µl steps and with constant mixing to 0.73 µmol (10 mg) of RNaseA (Fluka) in 1 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at room

temperature (RT) for 1 h. Excess reagent was then removed from the RNaseA conjugate by means of a PD-10 gel filtration column (Pharmacia) according to the manufacturer's instructions.

5

Unoccupied binding sites on the Immuno Stick surface were saturated by incubation with 1.2 ml of 2% w/v BSA in PBST (PBS with 0.1% v/v Tween 20) at RT for 2 h. After three short washes with in each case 1.2 ml of
10 PBST, the Immuno Stick was incubated in a mixture of 250 μ l of phagemid solution and 500 μ l of blocking buffer (2% w/v BSA in PBST) at RT for 1 h.

Unbound phagemids were removed by stripping off the
15 solution and washing the Immuno Stick eight times with in each case 950 μ l of PBST for 2 min. Finally, adsorbed phagemids were competitively eluted during a 15 minute incubation of the Immuno Stick with 950 μ l of a 2 mM solution of digoxigenin in PBS (0.742 mg of
20 digoxigenin (Fluka) were to this end dissolved in 19.2 μ l of DMF and added to 930.8 μ l of PBS).

The phagemids were propagated by heating 950 μ l of solution of the elution fraction obtained (between 10^6
25 and 10^8 colony-forming units, depending on the selection cycle) briefly to 37°C, mixing the solution with 4 ml of an exponentially growing culture of *E. coli* XL1-Blue ($OD_{550} = 0.5$) and incubated at 37°C, 200 rpm for 30 min. The phagemid-infected cells were
30 then sedimented (2 min, 4420 g, 4°C), resuspended in 800 μ l of fresh 2xYT medium and plated out on four agar plates containing LB/Amp medium (140 mm in diameter). After incubation at 32°C for 14 h, the colonies obtained in this way were scraped off the agar
35 plates with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension.

- Production and affinity concentration of phagemid particles were repeated by inoculating 50 ml of 2xYT/Amp medium prewarmed to 37°C with 0.2 to 1 ml of said suspension so that the cell density was 0.08 OD₅₅₀.
- 5 This culture was incubated at 37°C, 160 rpm to a cell density of OD₅₅₀ = 0.5, infected with 250 µl of VCS-M13 helper phage ($1.1 \cdot 10^{12}$ pfu/ml, Stratagene), and the procedure was continued as already described above.
- 10 The phagemids obtained from the first affinity concentration were used to carry out a series of eight further concentration cycles using Immuno Sticks which had been freshly coated with the digoxigenin-RNaseA conjugate. The phagemids obtained after the last
- 15 concentration cycle were again used for infecting *E. coli* XL1-Blue. The mixture of the colonies obtained was scraped off the agar plates using 2xYT/Amp medium and resuspended, as described above. This cell suspension was used to inoculate 50 ml of 2xYT/Amp
- 20 medium, and the phasmid DNA was isolated using the QIAprep Spin Miniprep kit (QIAGEN) according to the manufacturer's instructions.

In order to be able to produce the muteins of the

25 bilin-binding protein as a fusion protein with the Strep tag II and the albumin-binding domain, the gene cassette between the two *Bst*XI cleavage sites was subcloned from vector pBBP20 into vector pBBP22. A relevant section of the pBBP22 nucleic acid sequence is

30 represented, together with the encoded amino acid sequence, as SEQ ID NO. 9 in the sequence listing. The section starts with the *Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75.

35

For this purpose, the DNA isolated from the mixture of the *E. coli* colonies was cut with restriction enzyme *Bst*XI, and the smaller of the two fragments (335 bp) was purified by preparative agarose gel electrophoresis

as described above. In the same manner, pBBP22 vector DNA was cut with *Bst*XI and the larger of the two fragments (3545 bp) was isolated.

5 1.5 Weiss units of T4 DNA ligase (Promega) were added to 50 fmol of each of the two DNA fragments in a total volume of 20 μ l (30 mM Tris/HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP) and the mixture was incubated for ligation at 16°C overnight. 5 μ l of this ligation
10 mixture were used to transform 200 μ l of competent cells of *E. coli* strain TG1-F⁻ according to the CaCl₂ method (Sambrook et al., supra), and 2.2 ml of a cell suspension were obtained.

15 The transformants were then screened for production of muteins with binding activity for the digoxigenin group by means of a colony screening assay. For this purpose, a cut-to-fit hydrophilic PVDF membrane (Millipore, type GVWP, pore size 0.22 μ m) was marked at one position and
20 placed on an LB/Amp agar plate. 150 μ l of the cell suspension from the transformation mixture were plated out evenly on said membrane, and approx. 500 colonies were obtained. The plate was incubated in an incubator at 37°C for 7.5 h until the colonies were approx.
25 0.5 mm in diameter.

In the meantime, a hydrophobic membrane (Millipore, Immobilon P, pore size 0.45 μ m) which had likewise been cut to fit was wetted with PBS according to the
30 manufacturer's instructions and subsequently gently agitated in a solution of 10 mg/ml of human serum albumin (HSA, Sigma) in PBS at RT for 4 h. Remaining binding sites on the membrane were saturated by incubation with 3% w/v BSA, 0.5% v/v Tween 20 in PBS at
35 RT for 2 h. The membrane was washed with 20 ml of PBS for two times 10 min and then gently agitated in 10 ml of LB/Amp medium to which 200 μ g/l of anhydrotetracyclin had been added for 10 min. Said membrane was then marked at one position and placed on

a culture plate with LB/Amp agar which additionally contained 200 µg/l of anhydrotetracyclin.

5 The previously obtained hydrophilic membrane on which colonies had grown was then placed onto the hydrophobic membrane such that the two markings coincided. The culturing plate with the two membranes was incubated at 22°C for 15 h. During this phase, the particular
10 muteins were secreted by the colonies as fusion proteins and immobilized on the lower membrane by means of complex formation between the albumin-binding domain and the HSA.

15 Subsequently, the upper membrane containing the colonies was transferred to a fresh LB/Amp agar plate and stored at 4°C. The hydrophobic membrane was removed, washed with 20 ml of PBST for three times 10 min and then incubated in 10 ml of a 10 µg/ml solution of a conjugate of BSA with digoxigenin in PBST
20 for 1 h.

The conjugate of BSA (Sigma) and digoxigenin was prepared by adding a solution of 3.0 µmol (1.98 mg) of DIG-NHS in 25 µl of DMSO in µl steps and with constant
25 mixing to 300 nmol (19.88 mg) of BSA (Sigma) in 1.9 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at RT for 1 h and excess reagent was removed from the BSA conjugate by means of a PD-10 gel filtration column according to the
30 manufacturer's instructions.

In order to detect bound digoxigenin-BSA conjugate, the membrane was incubated, after washing twice in 20 ml of PBST, with 10 ml of anti-digoxigenin Fab-alkaline
35 phosphatase conjugate (Boehringer Mannheim, diluted 1:1000 in PBST) for 1 h. The membrane was then washed twice with 20 ml PBST and twice with 20 ml of PBST for in each case 5 min and gently agitated in AP buffer (0.1 M Tris/HCl pH 8.8, 0.1 M NaCl, 5 mM MgCl₂) for

10 min. For the chromogenic detection reaction, the membrane was incubated in 10 ml of AP buffer to which 30 µl of 5-bromo-4-chloro-3-indolyl phosphate, p-toluidinium salt (BCIP, Roth, 50 µg/ml in dimethylformamide) and 5 µl of Nitro Blue Tetrazolium (NBT, Sigma, 75 µg/ml in 70% v/v dimethylformamide) had been added, until at the positions of some of the colonies distinct color signals became visible. In this way, digoxigenin-binding activity of the bilin-binding protein muteins which had been produced in the form of fusion proteins with Strep tag and ABD by said colonies was detected.

Four colonies from the upper membrane, which caused a distinct color signal, were used for preparing cultures in LB/Amp medium of 4 ml in volume. Their plasmid DNA was isolated with the aid of the JETquick Plasmid Miniprep Spin kit (Genomed) according to the manufacturer's instructions, and the gene section coding for the mutein was subjected to sequence analysis. Sequence analysis was carried out with the aid of the T7 sequencing kit (Pharmacia) according to the manufacturer's instructions by using oligodeoxynucleotides SEQ ID NO. 10 and SEQ ID NO. 11. It was found in the process that all four plasmids studied carried the same nucleotide sequence. The corresponding gene product was denoted by DigA (SEQ ID NO. 12). The DigA nucleotide sequence was translated into the amino acid sequence and is represented in the sequence listing.

Example 2

Partial random mutagenesis of the DigA mutein and selection of muteins with improved binding affinity for digoxigenin

In order to improve the affinity between the DigA mutein and digoxigenin, which was determined as

295 ± 36 nM according to Example 3, the 6 amino acid positions 28, 31 and 34-37 in DigA were selected for a more substantial partial random mutagenesis.

5 Said positions were mutated by carrying out the PCR using a degenerated oligodeoxynucleotide primer. The amplification reaction was carried out in a total volume of 100 µl, with 2 ng of the vector pBBP22 plasmid DNA coding for DigA (SEQ ID NO. 12) being used
10 as matrix. The reaction mixture contained 50 pmol of the two primers SEQ ID NO. 13 and SEQ ID NO. 7 and also the other components according to the method described in Example 1. The PCR was carried out in 20 temperature cycles of 1 min at 94°C, 1 min at 65°C, and 1.5 min at
15 72°C, followed by a final incubation at 60°C for 5 min. The DNA fragment obtained was isolated by preparative agarose gel electrophoresis and then cut with *Bst*XI according to the manufacturer's instructions. The resulting DNA fragment of 335 bp in length was again
20 purified by preparative agarose gel electrophoresis.

PBBP24 vector DNA was cut with *Bst*XI accordingly and the 4028 bp fragment obtained was isolated. A relevant section of the pBBP24 nucleic acid sequence is
25 represented, together with the encoded amino acid sequence, as SEQ ID NO. 14 in the sequence listing. The section starts with the *Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75.
30 PBBP24 is virtually identical with pBBP20, and the BBP gene has been inactivated by means of appropriately introduced stop codons.

1.3 µg of the cleaved DNA fragment from the PCR and
35 16.0 µg of the pBBP24 fragment were incubated for ligation in the presence of 120 Weiss units of T4 DNA ligase (New England Biolabs) in a total volume of 600 µl (50 mM Tris/HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 50 µg/ml BSA) at 16°C for 18 h. The DNA was

then precipitated by adding 10 µg of yeast tRNA (Boehringer Mannheim), 25 µl of 5 M ammonium acetate and 100 µl of ethanol to in each case 24 µl of the ligation mixture. Incubation at -20°C for two weeks was followed by centrifugation (20 min, 16000 g, 4°C). The precipitate was washed in each case with 150 µl of ethanol (70% v/v, -20°C) and dried under reduced pressure. Finally, the DNA was taken up in 80 µl of TE/10.

10

E. coli XL1-Blue cells were transformed with the ligated DNA by electroporation according to the procedure described in Example 1, with in each case 40 µl of cell suspension of electrocompetent cells being mixed with 5 µl of the DNA solution in 16 mixtures. After electroporation, the cells were immediately diluted in 2 ml of fresh ice-cold SOC medium and agitated at 37°C and 200 rpm for 60 min.

20 168 ml of 2xYT medium and 200 µl of ampicillin (stock solution 100 mg/ml, final concentration 100 mg/l) were added to the combined suspensions. The total number of transformants obtained was estimated at $1.48 \cdot 10^9$ by plating out 100 µl of a $1:10^4$ dilution of the obtained cell suspension on agar plates. After incubation at 37°C and 160 rpm for 60 min, the transformants were infected with 4 ml of VCS-M13 helper phage ($6.3 \cdot 10^{11}$ pfu/ml, Stratagene) and agitated at 37°C and 160 rpm for a further 30 min. Subsequently, 400 µl of kanamycin (stock solution 35 mg/ml, final concentration 70 mg/l) were added, the incubator temperature was lowered to 26°C and, after 10 min, anhydrotetracyclin (100 µl of a 50 µg/ml stock solution in dimethylformamide, final concentration 25 µg/l) was added to induce gene expression. Finally, the phagemids were produced by incubating the culture at 26°C and 160 rpm for 7 h. The cells were removed and the phagemids purified by precipitation as described in Example 1.

Streptavidin-coated paramagnetic particles (Dynabeads M-280 Streptavidin, Dynal) were used together with a double conjugate of BSA with digoxigenin and biotin for affinity concentration from the library of phagemids which presented the partially mutated DigA mutein.

A double conjugate of BSA with digoxigenin and biotin was prepared by adding 1.5 μmol (0.99 mg) of DIG-NHS in 12.5 μl of DMSO and 1.5 μmol (0.68 mg) of D-biotinoyl- ϵ -aminocaproic acid N-hydroxysuccinimide ester (Boehringer Mannheim) in 12.5 μl of DMSO in μl steps and with constant mixing to 300 nmol (19.88 mg) of BSA in 1.9 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at RT for 1 h. Excess reagent was removed from the double conjugate via a PD-10 gel filtration column according to the manufacturer's instructions.

Digoxigenin-binding phagemids were concentrated by mixing 40 μl of a 0.5 μM solution of the double conjugate (33.5 $\mu\text{g}/\text{ml}$) in PBS with 260 μl of a solution of the freshly prepared phagemids (between $5 \cdot 10^{11}$ and $5 \cdot 10^{12}$ cfu/ml) and incubated at RT for 1 h so that the complex between the digoxigenin group and the muteins presented by the phagemids was able to form. This was followed by adding 100 μl of a solution of 8% w/v BSA, 0.4% v/v Tween 20 in PBS.

Parallel thereto, 100 μl of the commercially available suspension of paramagnetic particles were washed with three times 100 μl of PBS. Here, the particles were kept suspended for 1 min by rotating the 1.5 ml Eppendorf vessel and then collected at the wall of the Eppendorf vessel with the aid of a magnet, and the supernatant was stripped off. Unspecific binding sites were saturated by incubating the paramagnetic particles with 100 μl of 2% w/v BSA in PBST at RT for 1 h. After removing the supernatant, the mixture of double conjugate and phagemids was added to the paramagnetic

particles, and the particles were resuspended and incubated at RT for 10 min. Finally, free biotin-binding sites of Streptavidin were saturated by adding 10 μ l of a 4 μ M D-dethiobiotin (Sigma) solution in PBS to the mixture and incubating said mixture at RT for 5 min. This procedure also prevented the Strep tag II as part of the fusion protein of the muteins and the phage coat protein pIII fragment from being able to form a complex with Streptavidin.

10

Unbound phagemids were removed by washing the paramagnetic particles with eight times 1 ml of fresh PBST with the addition of 1 mM D-dethiobiotin, the particles were collected with the aid of the magnet and the supernatant was stripped off. The bound phagemids were eluted by incubating the resuspended particles in 950 μ l of 0.1 M glycine/HCl pH 2.2 for 15 minutes. After collecting the particles on the magnet, the supernatant was again stripped off and this was immediately followed by neutralizing the pH of said solution by addition of 140 μ l of 0.5 M Tris.

The phagemids were propagated by mixing the elution fraction obtained, according to the procedure in Example 1, with 4 ml of an exponentially growing culture of *E. coli* XL1-Blue ($OD_{550} = 0.5$) and incubating at 37°C, 200 rpm for 30 min. The phagemid-infected cells were then sedimented (2 min, 4420 g, 4°C), resuspended in 800 μ l of fresh 2xYT medium and plated out on four agar plates containing LB/Amp medium (140 mm in diameter). After incubation at 32°C for 14 h, the colonies obtained in this way were scraped off the agar plates with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension.

Production and affinity concentration of phagemid particles were repeated by inoculating 50 ml of

2xYT/Amp medium prewarmed to 37°C with 0.2 to 1 ml of said suspension so that the cell density was 0.08 OD₅₅₀. This culture was incubated at 37°C, 160 rpm to a cell density of OD₅₅₀ = 0.5 and infected with 300 µl of VCS-M13 helper phage ($6.3 \cdot 10^{11}$ pfu/ml, Stratagene). The affinity selection was then repeated using the paramagnetic particles and the digoxigenin/biotin double conjugate under the abovementioned conditions. A total of 4 selection cycles were carried out in this way.

The phagemids obtained after the last concentration cycle were again used for infecting *E. coli* XL1-Blue. The mixture of the obtained colonies which had been scraped off the agar plates using 2xYT/Amp medium and had been resuspended, as described above, was used to inoculate 50 ml of 2xYT/Amp medium, and phasmid DNA was isolated using the QIAprep spin miniprep kit (QIAGEN) according to the manufacturer's instructions.

Subsequently, the gene cassette between the two *Bst*XI cleavage sites was subcloned, as in Example 1, from vector pBBP24 into vector pBBP22, and competent cells of *E. coli* strain TG1-F⁻ were transformed according to the CaCl₂ method. Finally, the transformants were, again according to Example 1, screened for production of muteins with binding activity for the digoxigenin group by means of the colony screening assay.

Seven of the colonies showing a strong signal intensity in the colony screening assay were cultured. Their plasmid DNA was isolated with the aid of the plasmid miniprep spin kit (Genomed) according to the manufacturer's instructions, and the gene section coding for the mutein was subjected to sequence analysis as in Example 1. It was found in the process that all plasmids studied had different sequences. After translating the nucleotide sequences into amino acid sequences, six of the seven variants studied had an amber stop codon at amino acid position 28. However,

this stop codon was at least partially suppressed when choosing suitable amber-suppressor strains such as, for example, *E. coli* XL1-Blue or TG1-F⁻ and instead translated as glutamine. Thus a full-length functional protein was produced both during affinity concentration and in the colony screening assay.

As the only mutein without an amber stop codon among the muteins found, the mutein with SEQ ID NO. 15 was particularly well suited to bacterial production. Consequently, this mutein, also denoted by DigA16, was characterized in more detail with regard to its ability to bind to the digoxigenin group.

Example 3

Production of the DigA and DigA16 muteins and determination of their affinity for digoxigenin and derivatives thereof by fluorescence titration

The bilin-binding protein muteins obtained from the previous examples were preparatively produced by subcloning the coding gene section between the two *Bst*XI cleavage sites from the type pBBP22 vector into the expression plasmid pBBP21. The plasmid thus obtained coded for a fusion protein of the OmpA signal sequence, followed by the mutein and the Strep tag II affinity tag.

A relevant section of the pBBP21 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO. 16 in the sequence listing. The section starts with the *Xba*I cleavage site and ends with a hexanucleotide which was obtained by ligating a blunt strand end with a filled-up *Hind*III strand end, with the loss of the original *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75.

For subcloning, the plasmid DNA coding for the relevant murein was cut with restriction enzyme *Bst*XI, and the smaller of the two fragments (335 bp) was purified by preparative agarose gel electrophoresis as described in Example 1. In the same manner, pBBP21 vector DNA was cut with *Bst*XI, and the larger of the two fragments (4132 bp) was isolated.

1.5 Weiss units of T4 DNA ligase (Promega) were added to 50 fmol of each of the two DNA fragments in a total volume of 20 μ l (30 mM Tris/HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP) and the mixture was incubated for ligation at 16°C for 16 h. 5 μ l of the ligation mixture were then used to transform *E. coli* JM83 (Yanisch-Perron et al., Gene 33 (1985), 103-119) according to the CaCl₂ method, and 2.2 ml of a cell suspension were obtained. 100 μ l of this suspension were plated out on an agar plate containing LB/Amp medium and incubated at 37°C for 14 h.

The protein was produced by selecting one of the obtained single colonies, using it to inoculate a 50 ml preculture (LB/Amp medium) and incubating said preculture at 30°C and 200 rpm overnight. 40 ml of the preculture were then transferred by inoculating 2 l of LB/Amp medium in a 5 l Erlenmeyer flask, followed by incubating the culture at 22°C and 200 rpm. At a cell density of OD₅₅₀ = 0.5, gene expression was induced by adding 200 μ g/l anhydrotetracyclin (200 μ l of a 2 mg/ml stock solution in DMF), followed by agitating at 22°C, 200 rpm for a further 3 h.

The cells were removed by centrifugation (15 min, 4 420 g, 4°C) and, after removing the supernatant, resuspended in 20 ml of periplasm lysis buffer (100 mM Tris/HCl pH 8.0, 500 mM sucrose, 1 mM EDTA) with cooling on ice. After incubation on ice for 30 min, the spheroplasts were removed in two successive centrifugation steps (15 min, 4 420 g, 4°C and 15 min,

30 000 g, 4°C). The periplasmic protein extract obtained in this way was dialyzed against SA buffer (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA), sterile-filtered and used for chromatographic purification.

Purification was carried out by means of the Strep tag II affinity tag (Schmidt and Skerra, Protein Eng. 6 (1993), 109-122) fused to the C terminus of the muteins. In the present case, Streptavidinmutein "1" was used (Voss and Skerra, Protein Eng. 10 (1997), 975-982), which was coupled to activated Sepharose (via 5 mg/ml immobilized Streptavidin, based on the bett volume of the matrix).

A chromatography column packed with 2 ml of said material was equilibrated at 4°C and a flow rate of 20 ml/h with 10 ml of SA buffer. The chromatography was monitored by measuring absorption of the eluate at 280 nm in a flow-through photometer. Application of the periplasmic protein extract was followed by washing with SA buffer until the base line was reached. Bound mutein was then eluted with 10 ml of a solution of 2.5 mM D-dethiobiotin (Sigma) in SA buffer. The fractions containing the purified mutein were checked by means of SDS polyacrylamide gel electrophoresis (Fling and Gregerson, Anal. Biochem. 155 (1986), 83-88) and combined. The protein yields were between 200 µg and 800 µg per 2 l culture.

The ligand binding properties of muteins DigA, DigA16 and also of the recombinant bilin-binding protein (SEQ ID NO: 16) were determined by means of the method of fluorescence titration.

In this case, the decrease in intrinsic tyrosine and/or tryptophan fluorescence of the protein forming a complex with the ligand was measured. The measurements were carried out in a fluorimeter, type LS 50 B (Perkin

Elmer) at an excitation wavelength of 295 nm (slit width 4 nm) and an emission wavelength of 345 nm (slit width 6 nm). The ligands used were digoxigenin (Fluka), digoxin (Fluka), digitoxigenin (Fluka), digitoxin (Fluka), testosterone (Sigma), ouabain (Fluka), and 4-aminofluorescein (Fluka). The ligands showed no significant intrinsic fluorescence or absorption at the stated wavelength.

10 The buffer system used was PBS with the addition of 1 mM EDTA. The solution of the relevant purified mutein was dialyzed four times against this buffer and adjusted to a concentration of 1 μ M by dilution. All solutions used were sterile-filtered (Filtropur S 15 0.45 μ m, Sarstedt). The concentration was determined by means of absorption at 280 nm using calculated extinction coefficients of 53 580 $M^{-1} cm^{-1}$ for DigA and DigA16 (Wisconsin Software Package, Genetics Computer Group). For Bbp, the calculated extinction coefficient 20 of 54 150 $M^{-1} cm^{-1}$, corrected in the presence of guanidinium chloride according to Gill and von Hippel (Anal. Biochem. 182 (1989), 319-326) was used.

For the measurement, 2 ml of the mutein solution were 25 introduced into a quartz cuvette equipped with a magnetic stirrer bar and thermally equilibrated at 25°C in the sample holder of the photometer. Then a total of 40 μ l of a 100 μ M to 500 μ M solution of the ligand in the same buffer were pipetted in steps of from 1 μ l to 30 4 μ l. The dilution of the introduced protein solution by altogether no more than 2%, which took place in the process, was not taken into account in the subsequent evaluation of the data. After each titration step, the equilibrium was allowed to form by incubating with 35 stirring for 1 min, and the fluorescence signal was measured as average over 10 s. After subtracting the fluorescence value of the buffer, the signals were normalized to an initial value of 100%.

The thus obtained data of a titration series were fitted by nonlinear regression with the aid of the computer program Kaleidagraph (Abelbeck Software) according to the following formula

5

$$F = ([P]_t - [L]_t - K_d) \frac{f_P}{2} + ([P]_t + [L]_t + K_d) \frac{f_{PL}}{2} + (f_P - f_{PL}) \sqrt{\frac{([P]_t + [L]_t + K_d)^2}{4} - [P]_t [L]_t}$$

Here, F means the normalized fluorescence intensity and [L]_t the total ligand concentration in the particular titration step. [P]_t as mutein concentration, f_{PL} as fluorescence coefficient of the mutein-ligand complex and K_d as the thermodynamic dissociation constant of said complex were fitted as free parameters to the normalized data.

15

Fig. 1 represents graphically the results of the fluorescence titrations of the DigA16 mutein with the ligands digoxigenin, digitoxigenin and ouabain. It turns out that digitoxigenin is bound even tighter than digoxigenin, while no binding is observed for ouabain.

The values resulting from the fluorescence titrations for the dissociation constants of the complexes of the bilin-binding protein muteins and the various ligands are summarized in the following table:

<u>Bbp variant</u>	<u>Ligand</u>	<u>K_d [nM]</u>
Bbp:	digoxigenin	-*
30 DigA:	digoxigenin	295 ± 37
	digoxin	200 ± 34
DigA16:	digoxigenin	30.2 ± 3.6
	digoxin	31.1 ± 3.2
35	digitoxigenin	2.8 ± 2.7
	digitoxin	2.7 ± 2.0
	ouabain	-*

testosterone	-*
4-aminofluorescein	-*

* no detectable binding activity

5

Example 4

Preparation of fusion proteins of the DigA16 mutein and
bacterial alkaline phosphatase and use for detecting
10 digoxigenin groups in an ELISA and in a Western blot

In order to produce two different fusion proteins of
the DigA16 mutein and bacterial alkaline phosphatase
(PhoA) with different arrangement of the partners
15 within the polypeptide chain, the two expression
plasmids pBBP27 and pBBP29 were constructed by using
the molecular-biological methods familiar to the
skilled worker.

20 pBB27 codes for a fusion protein of PhoA including the
signal sequence thereof, a short peptide linker having
the amino acid sequence Pro-Pro-Ser-ala, the sequence
corresponding to the mature DigA16 mutein and the Strep
tag II. A relevant section of the pBBP27 nucleic acid
25 sequence is represented, together with the encoded
amino acid sequence, as SEQ ID NO: 17 in the sequence
listing. The section begins with the *Xba*I cleavage site
and ends with the *Hind*III cleavage site. The vector
elements outside this region are identical to vector
30 pBBP21.

pBB29 codes for a fusion protein of DigA16 with
preceding OmpA signal sequence, followed by the peptide
sequence for Strep tag II, a sequence of 5 glycine
35 residues and the mature PhoA sequence without the N-
terminal amino acid arginine. A relevant section of the
pBBP29 nucleic acid sequence is represented, together
with the encoded amino acid sequence, as SEQ ID NO: 18
in the sequence listing. The section begins with the

*Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pBBP21.

5 Both plasmids additionally code for the bacterial protein disulfide isomerase DsbC on a separate cistron located in 3' direction. The plasmids are shown diagrammatically in **Fig. 2**.

10 The fusion proteins encoded by plasmids pBBP27 and pBBP29 were produced analogously to the method for preparing the simple muteins, described in example 3. In order to avoid complexing the metal ions from the active center of PhoA, lysis of the bacterial periplasm
15 was carried out using EDTA-free lysis buffer. Polymyxin B sulfate (2 mg/ml, Sigma) was added to the buffer as an agent destabilizing the outer cell membrane. All other buffers used for purification were likewise EDTA-free.

20

The fusion proteins purified by affinity chromatography by means of the Strep tag II were dialyzed against PBS buffer overnight. The fusion protein yields were between 100 and 200 µg per 2 l of culture medium. The
25 purity of the fusion proteins obtained was checked by SDS polyacrylamide gel electrophoresis, according to example 3, and determined at 90-95%. Subsequently, the fusion proteins were used for directly detecting conjugates of the digoxigenin group with various
30 proteins both in a sandwich ELISA and in a Western blot.

While the conjugates used of digoxigenin with RNaseA and BSA were prepared according to example 1, a
35 conjugate of digoxigenin with ovalbumin (Sigma) was prepared by adding 1.5 µmol (0.99 mg) DIG-NHS in 25 µl of DMSO in µl steps and with constant mixing to 300 nmol (13.5 mg) of ovalbumin in 1.9 ml of 5% sodium hydrogen carbonate. The mixture was incubated with

stirring at RT for 1 h. Excess reagent was removed from the ovalbumin conjugate via a PD-10 gel filtration column according to the manufacturer's instructions.

5 For detecting digoxigenin groups in a sandwich ELISA, the wells in in each case two vertical columns of a microtiter plate (ELISA strips, 2x8 well with high binding capacity, F type, Greiner) were filled in each case with 100 μ l of a 100 μ g/ml solution of the BSA-
10 digoxigenin conjugate or the ovalbumin-digoxigenin conjugate in PBS and incubated at RT overnight. As a control, the wells of a fifth vertical row of the microtiter plate were filled with 100 μ l of a 100 μ g/ml solution of nonconjugated BSA (Sigma) in PBS and
15 likewise incubated at RT overnight. After removing the solution, unoccupied binding sites were saturated with 200 μ l of a solution of 2% w/v BSA in PBST for 2 h. After washing three times with PBST, 50 μ l of a 1 μ M solution of the purified fusion protein were in each
20 case introduced into the first well of a row, and the Tween concentration was adjusted to 0.1% v/v by adding 1 μ l of a solution of 5% v/v Tween. The subsequent wells in each row were initially charged with 50 μ l of PBST. Then, 50 μ l of the purified fusion protein were
25 pipetted in each case into the second well, mixed and, starting therefrom, 1:2 dilutions were prepared stepwise in the other wells of the vertical row. After incubation at RT for 1 h, the wells were washed twice with PBST and twice with PBS. The fusion proteins bound
30 to the digoxigenin groups were finally detected by means of alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate. For this purpose, 100 μ l of a solution of 0.5 mg/ml p-nitrophenyl phosphate (Amresco) in AP buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris/HCl
35 pH 8.8) were introduced into the wells and product formation was monitored by measuring absorption at 405 nm in a SpectraMax 250 photometer (Molecular Devices).

Fig. 3 shows the result of this measurement. According to this, the digoxigenin group is recognized both as conjugate with BSA and as conjugate with ovalbumin, leading to the conclusion that binding by the DigA16
5 mutein is context-independent. Furthermore, both fusion proteins are active both with regard to the binding function for the digoxigenin group and enzymatically and produce, despite their different structure, almost identical signals.

10

The fusion proteins encoded by vectors pBBP27 and pBBP29 were used in a Western blot by first fractionating 5 μ l of a protein mixture in PBS, whose concentration of digoxigenin-BSA conjugate,
15 digoxigenin-ovalbumin conjugate and digoxigenin-RNaseA conjugate was simultaneously in each case 100 μ g/ml, and also 5 μ l of a protein mixture in PBS, whose concentration of underivatized BSA, ovalbumin and RNaseA likewise was simultaneously in each case
20 100 μ g/ml, by SDS polyacrylamide gel electrophoresis. The protein mixture was then transferred to nitrocellulose by electrotransfer (Blake et al., Anal. Biochem. 136 (1984), 175-179). The membrane was then washed in 10 ml of PBST for three times 5 min and
25 incubated with 10 ml of a 0.5 μ M solution of in each case one of the two fusion proteins for 1 h. The membrane was then washed in 10 ml PBST for two times 5 min and in 10 ml of PBS for two times 5 min and finally gently agitated in 10 ml of AP buffer for
30 10 min. For the chromogenic detection reaction, the membrane was incubated in 10 ml of AP buffer to which 30 μ l BCIP (50 μ g/ml in dimethylformamide) and 5 μ l NBT (75 μ g/ml in 70% v/v dimethylformamide) had been added, and in this way bound fusion protein was detected.

35

Fig. 4 shows the result of this detection method. It turns out again that binding of the digoxigenin group by the two fusion proteins is independent of the carrier protein and that both fusion proteins achieve

comparable signal intensities. The same carrier proteins cause no signal whatsoever if they are not conjugated with the digoxigenin group.

DE 199 26 068 C1

SEQUENCE LISTING

GENERAL INFORMATION:

APPLICANT:

NAME: Prof. Dr. Arne Skerra
STREET: Max-Lehner-Straße 18
CITY: Freising
COUNTRY: Germany
ZIP: 85354
TELEPHONE: 08161-714351
TELEFAX: 08161-714352

TITLE OF THE INVENTION: Muteins of the bilin-binding protein

NUMBER OF SEQUENCES: 18

COMPUTER READABLE FORM:

MEDIUM TYPE: Floppy disk
COMPUTER: IBM PC compatible
OPERATING SYSTEM: PC-DOS/MS-DOS
SOFTWARE: Microsoft Word, format: Text

CURRENT APPLICATION DATA:

APPLICATION NUMBER: not yet known
FILING DATE: not yet known

INFORMATION FOR SEQ ID NO:1:

SEQUENCE CHARACTERISTICS:

LENGTH: 1219 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: fragment of phasmid pBBP20

FEATURE:

NAME/KEY: signal peptide
LOCATION: (22..84)

FEATURE:

NAME/KEY: mature peptide
LOCATION: (85..1209)
OTHER INFORMATION:
/Product = "fusion protein composed of bilin-binding protein
Strep-tag II and fragment of phage envelope
protein pIII"
/Codon = (sequence: "TAG", amino acid:Gln)

FEATURE:

NAME/KEY: coding sequence
LOCATION: (85..606)
OTHER INFORMATION:
/Product = "mature bilin-binding protein"

NAME/KEY: coding sequence
LOCATION: (607..636)
OTHER INFORMATION:
/Product = "Strep-tag II affinity tag"

NAME/KEY: coding sequence
LOCATION: (637..639)
OTHER INFORMATION:
/Other = "amber stop codon"

NAME/KEY: coding sequence
LOCATION: (640..1209)
OTHER INFORMATION:
/Product = "amino acids 217-406 of envelope protein pIII"

TCTAGTTAAC	GAGGGCAAAA	A	ATG	AAA	AAG	ACA	GCT	ATC	GCG	ATT					45
			Met	Lys	Lys	Thr	Ala	Ile	Ala	Ile					
			-21	-20						-15					
GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC GAC GTG	90														
Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Asp Val															
			-10					-5				-1	1		
TAC CAC GAC GGT GCC TGT CCC GAA GTC AAG CCA GTC GAC AAC TTC	135														
Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro Val Asp Asn Phe															
		5					10					15			
GAC TGG TCC CAG TAC CAT GGT AAA TGG TGG GAA GTC GCC AAA TAC	180														
Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Glu Val Ala Lys Tyr															
		20					25					30			
CCC AAC TCA GTT GAG AAG TAC GGA AAG TGC GGA TGG GCT GAG TAC	225														
Pro Asn Ser Val Glu Lys Tyr Gly Lys Cys Gly Trp Ala Glu Tyr															
		35					40					45			
ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG AAC TAC CAC GTA ATC	270														
Thr Pro Glu Gly Lys Ser Val Lys Val Ser Asn Tyr His Val Ile															
		50					55					60			
CAC GGC AAG GAA TAC TTT ATT GAA GGA ACT GCC TAC CCA GTT GGT	315														
His Gly Lys Glu Tyr Phe Ile Glu Gly Thr Ala Tyr Pro Val Gly															
		65					70					75			
GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC CTG ACT TAC GGA GGT	360														
Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Leu Thr Tyr Gly Gly															
		80					85					90			
GTC ACC AAG GAG AAC GTA TTC AAC GTA CTC TCC ACT GAC AAC AAG	405														
Val Thr Lys Glu Asn Val Phe Asn Val Leu Ser Thr Asp Asn Lys															
		95					100					105			

DE 199 26 068 C1

AAC	TAC	ATC	ATC	GGA	TAC	TAC	TGC	AAA	TAC	GAC	GAG	GAC	AAG	AAG	450
Asn	Tyr	Ile	Ile	Gly	Tyr	Tyr	Cys	Lys	Tyr	Asp	Glu	Asp	Lys	Lys	
		110					115					120			
GGA	CAC	CAA	GAC	TTC	GTC	TGG	GTG	CTC	TCC	AGA	AGC	ATG	GTC	CTT	495
Gly	His	Gln	Asp	Phe	Val	Trp	Val	Leu	Ser	Arg	Ser	Met	Val	Leu	
		125					130					135			
ACT	GGT	GAA	GCC	AAG	ACC	GCT	GTC	GAG	AAC	TAC	CTT	ATC	GGC	TCC	540
Thr	Gly	Glu	Ala	Lys	Thr	Ala	Val	Glu	Asn	Tyr	Leu	Ile	Gly	Ser	
		140					145					150			
CCA	GTA	GTC	GAC	TCC	CAG	AAA	CTG	GTA	TAC	AGT	GAC	TTC	TCT	GAA	585
Pro	Val	Val	Asp	Ser	Gln	Lys	Leu	Val	Tyr	Ser	Asp	Phe	Ser	Glu	
		155					160					165			
GCC	GCC	TGC	AAG	GTC	AAC	AAT	AGC	AAC	TGG	TCT	CAC	CCG	CAG	TTC	630
Ala	Ala	Cys	Lys	Val	Asn	Asn	Ser	Asn	Trp	Ser	His	Pro	Gln	Phe	
		170					175					180			
GAA	AAA	TAG	GCT	GGC	GGC	GGC	TCT	GGT	GGT	GGT	TCT	GGC	GGC	GGC	675
Glu	Lys	Gln	Ala	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Ser	Gly	Gly	Gly	
		185					190					195			
TCT	GAG	GGT	GGT	GGC	TCT	GAG	GGT	GGC	GGT	TCT	GAG	GGT	GGC	GGC	720
Ser	Glu	Gly	Gly	Gly	Ser	Glu	Gly	Gly	Gly	Ser	Glu	Gly	Gly	Gly	
		200					205					210			
TCT	GAG	GGA	GGC	GGT	TCC	GGT	GGT	GGC	TCT	GGT	TCC	GGT	GAT	TTT	765
Ser	Glu	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Ser	Gly	Ser	Gly	Asp	Phe	
		215					220					225			
GAT	TAT	GAA	AAG	ATG	GCA	AAC	GCT	AAT	AAG	GGG	GCT	ATG	ACC	GAA	810
Asp	Tyr	Glu	Lys	Met	Ala	Asn	Ala	Asn	Lys	Gly	Ala	Met	Thr	Glu	
		230					235					240			
AAT	GCC	GAT	GAA	AAC	GCG	CTA	CAG	TCT	GAC	GCT	AAA	GGC	AAA	CTT	855
Asn	Ala	Asp	Glu	Asn	Ala	Leu	Gln	Ser	Asp	Ala	Lys	Gly	Lys	Leu	
		245					250					255			
GAT	TCT	GTC	GCT	ACT	GAT	TAC	GGT	GCT	GCT	ATC	GAT	GGT	TTC	ATT	900
Asp	Ser	Val	Ala	Thr	Asp	Tyr	Gly	Ala	Ala	Ile	Asp	Gly	Phe	Ile	
		260					265					270			
GGT	GAC	GTT	TCC	GGC	CTT	GCT	AAT	GGT	AAT	GGT	GCT	ACT	GGT	GAT	945
Gly	Asp	Val	Ser	Gly	Leu	Ala	Asn	Gly	Asn	Gly	Ala	Thr	Gly	Asp	
		275					280					285			
TTT	GCT	GGC	TCT	AAT	TCC	CAA	ATG	GCT	CAA	GTC	GGT	GAC	GGT	GAT	990
Phe	Ala	Gly	Ser	Asn	Ser	Gln	Met	Ala	Gln	Val	Gly	Asp	Gly	Asp	
		290					295					300			
AAT	TCA	CCT	TTA	ATG	AAT	AAT	TTC	CGT	CAA	TAT	TTA	CCT	TCC	CTC	1035
Asn	Ser	Pro	Leu	Met	Asn	Asn	Phe	Arg	Gln	Tyr	Leu	Pro	Ser	Leu	
		305					310					315			

DE 199 26 068 C1

CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA	1080
Pro Gln Ser Val Glu Cys Arg Pro Phe Val Phe Gly Ala Gly Lys	
320 325 330	
CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA AAC TTA TTC CGT	1125
Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg	
335 340 345	
GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA	1170
Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val	
350 355 360	
TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT	1209
Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser	
365 370 375	
TAATAAGCTT	1219

INFORMATION FOR SEQ ID NO:2:

SEQUENCE CHARACTERISTICS:

LENGTH: 64 bases
 TYPE: nucleic acid
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:2

CCATGGTAAA TGGTGGAAG TCGCCAAATA CCCCNNKNMS NNSNNKAAGT	50
ACGGAAAGTG CGGA	64

INFORMATION FOR SEQ ID NO:3:

SEQUENCE CHARACTERISTICS:

LENGTH: 71 bases
 TYPE: nucleic acid
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGTAGGCGG TACCTTCSNN AAAGTATTCC TTGCCGTGGA TTACMNGTA	50
SNNCGAACT TTGACACTCT T	71

INFORMATION FOR SEQ ID NO:4:

SEQUENCE CHARACTERISTICS:

LENGTH: 74 bases
 TYPE: nucleic acid
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: synthetic oligodeoxynucleotide

DE 199 26 068 C1

SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCAAGATTGG AAAGATCTAC CACAGCNNSA CTNNKGGAGG TNNSACCVVS 50
GAGNNKGTAT TCAACGTACT CTCC 74

INFORMATION FOR SEQ ID NO:5:

SEQUENCE CHARACTERISTICS:

LENGTH: 78 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCTGGAGAGC ACCCAGACMN NGTCSNNGTG TCCCTTCTTG TCCTCGTCGT 50
ASNNGCAMNN GTATCCGATG ATGTAGTT 78

INFORMATION FOR SEQ ID NO:6:

SEQUENCE CHARACTERISTICS:

LENGTH: 36 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTTCGACTGG TCCCAGTACC ATGGTAAATG GTGGGA 36

INFORMATION FOR SEQ ID NO:7:

SEQUENCE CHARACTERISTICS:

LENGTH: 37 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:7:

CACCAGTAAG GACCATGCTT CTGGAGAGCA CCCAGAC 37

INFORMATION FOR SEQ ID NO:8:

SEQUENCE CHARACTERISTICS:

LENGTH: 46 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

DE 199 26 068 C1

SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGATCTTTCC AATCTTGGAG TCACCAACTG GGTAGGCGGT ACCTTC 46

INFORMATION FOR SEQ ID NO:9:

SEQUENCE CHARACTERISTICS:

LENGTH: 793 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: fragment of plasmid pBBP22

FEATURE:

NAME/KEY: signal peptide
LOCATION: (22..84)

FEATURE:

NAME/KEY: mature peptide
LOCATION: (85..783)
OTHER INFORMATION:
/Product = "fusion protein composed of bilin-binding protein, Strep-tag II and albumin-binding domain"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (85..606)
OTHER INFORMATION:
/Product = "mature bilin-binding protein"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (607..636)
OTHER INFORMATION:
/Product = "Strep-tag II affinity tag"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (637..783)
OTHER INFORMATION:
/Product = "albumin-binding domain of protein G"

SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCTAGATAAC	GAGGGCAAAA	A	ATG	AAA	AAG	ACA	GCT	ATC	GCG	ATT	45				
			Met	Lys	Lys	Thr	Ala	Ile	Ala	Ile					
			-21	-20					-15						
GCA	GTG	GCA	CTG	GCT	GGT	TTC	GCT	ACC	GTA	GCG	CAG	GCC	GAC	GTG	90
Ala	Val	Ala	Leu	Ala	Gly	Phe	Ala	Thr	Val	Ala	Gln	Ala	Asp	Val	
			-10				-5				-1	1			
TAC	CAC	GAC	GGT	GCC	TGT	CCC	GAA	GTC	AAG	CCA	GTC	GAC	AAC	TTC	135
Tyr	His	Asp	Gly	Ala	Cys	Pro	Glu	Val	Lys	Pro	Val	Asp	Asn	Phe	
			5				10					15			

DE 199 26 068 C1

GAC	TGG	TCC	CAG	TAC	CAT	GGT	AAA	TGG	TGG	GAA	GTC	GCC	AAA	TAC	180
Asp	Trp	Ser	Gln	Tyr	His	Gly	Lys	Trp	Trp	Glu	Val	Ala	Lys	Tyr	
		20					25					30			
CCC	AAC	TCA	GTT	GAG	AAG	TAC	GGA	AAG	TGC	GGA	TGG	GCT	GAG	TAC	225
Pro	Asn	Ser	Val	Glu	Lys	Tyr	Gly	Lys	Cys	Gly	Trp	Ala	Glu	Tyr	
		35					40					45			
ACT	CCT	GAA	GGC	AAG	AGT	GTC	AAA	GTT	TCG	AAC	TAC	CAC	GTA	ATC	270
Thr	Pro	Glu	Gly	Lys	Ser	Val	Lys	Val	Ser	Asn	Tyr	His	Val	Ile	
		50					55					60			
CAC	GGC	AAG	GAA	TAC	TTT	ATT	GAA	GGA	ACT	GCC	TAC	CCA	GTT	GGT	315
His	Gly	Lys	Glu	Tyr	Phe	Ile	Glu	Gly	Thr	Ala	Tyr	Pro	Val	Gly	
		65					70					75			
GAC	TCC	AAG	ATT	GGA	AAG	ATC	TAC	CAC	AGC	CTG	ACT	TAC	GGA	GGT	360
Asp	Ser	Lys	Ile	Gly	Lys	Ile	Tyr	His	Ser	Leu	Thr	Tyr	Gly	Gly	
		80					85					90			
GTC	ACC	AAG	GAG	AAC	GTA	TTC	AAC	GTA	CTC	TCC	ACT	GAC	AAC	AAG	405
Val	Thr	Lys	Glu	Asn	Val	Phe	Asn	Val	Leu	Ser	Thr	Asp	Asn	Lys	
		95					100					105			
AAC	TAC	ATC	ATC	GGA	TAC	TAC	TGC	AAA	TAC	GAC	GAG	GAC	AAG	AAG	450
Asn	Tyr	Ile	Ile	Gly	Tyr	Tyr	Cys	Lys	Tyr	Asp	Glu	Asp	Lys	Lys	
		110					115					120			
GGA	CAC	CAA	GAC	TTC	GTC	TGG	GTG	CTC	TCC	AGA	AGC	ATG	GTC	CTT	495
Gly	His	Gln	Asp	Phe	Val	Trp	Val	Leu	Ser	Arg	Ser	Met	Val	Leu	
		125					130					135			
ACT	GGT	GAA	GCC	AAG	ACC	GCT	GTC	GAG	AAC	TAC	CTT	ATC	GGC	TCC	540
Thr	Gly	Glu	Ala	Lys	Thr	Ala	Val	Glu	Asn	Tyr	Leu	Ile	Gly	Ser	
		140					145					150			
CCA	GTA	GTC	GAC	TCC	CAG	AAA	CTG	GTA	TAC	AGT	GAC	TTC	TCT	GAA	585
Pro	Val	Val	Asp	Ser	Gln	Lys	Leu	Val	Tyr	Ser	Asp	Phe	Ser	Glu	
		155					160					165			
GCC	GCC	TGC	AAG	GTC	AAC	AAT	AGC	AAC	TGG	TCT	CAC	CCG	CAG	TTC	630
Ala	Ala	Cys	Lys	Val	Asn	Asn	Ser	Asn	Trp	Ser	His	Pro	Gln	Phe	
		170					175					180			
GAA	AAA	CCA	GCT	AGC	CTG	GCT	GAA	GCT	AAA	GTT	CTG	GCT	AAC	CGT	675
Glu	Lys	Pro	Ala	Ser	Leu	Ala	Glu	Ala	Lys	Val	Leu	Ala	Asn	Arg	
		185					190					195			
GAA	CTG	GAC	AAA	TAC	GGT	GTT	TCC	GAC	TAC	TAC	AAA	AAC	CTC	ATC	720
Glu	Leu	Asp	Lys	Tyr	Gly	Val	Ser	Asp	Tyr	Tyr	Lys	Asn	Leu	Ile	
		200					205					210			
AAC	AAC	GCT	AAA	ACC	GTT	GAA	GGT	GTT	AAA	GCT	CTG	ATC	GAC	GAA	765
Asn	Asn	Ala	Lys	Thr	Val	Glu	Gly	Val	Lys	Ala	Leu	Ile	Asp	Glu	
		215					220					225			

DE 199 26 068 C1

ATT CTC GCA GCA CTG CCG TAATAAGCTT
Ile Leu Ala Ala Leu Pro
230

793

INFORMATION FOR SEQ ID NO:10:

SEQUENCE CHARACTERISTICS:

LENGTH: 17 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:10:

GACGGTGCCT GTCCCGA 17

INFORMATION FOR SEQ ID NO:11:

SEQUENCE CHARACTERISTICS:

LENGTH: 17 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:11:

GACTACTGGG GAGCCGA 17

INFORMATION FOR SEQ ID NO:12:

SEQUENCE CHARACTERISTICS:

LENGTH: 522
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: coding sequence of mutein Diga

FEATURE:

NAME/KEY: coding sequence
LOCATION: (1..522)
OTHER INFORMATION:
/Product = "mutein without fusion parts"

SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAC	GTG	TAC	CAC	GAC	GGT	GCC	TGT	CCC	GAA	GTC	AAG	CCA	GTC	GAC	45
Asp	Val	Tyr	His	Asp	Gly	Ala	Cys	Pro	Glu	Val	Lys	Pro	Val	Asp	
1				5					10					15	
AAC	TTC	GAC	TGG	TCC	CAG	TAC	CAT	GGT	AAA	TGG	TGG	GAA	GTC	GCC	90
Asn	Phe	Asp	Trp	Ser	Gln	Tyr	His	Gly	Lys	Trp	Trp	Glu	Val	Ala	
				20					25					30	

DE 199 26 068 C1

AAA TAC CCC CAT CAC GAG CGG AAG TAC GGA AAG TGC GGA TGG GCT	135
Lys Tyr Pro His His Glu Arg Lys Tyr Gly Lys Cys Gly Trp Ala	
35 40 45	
GAG TAC ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG CGC TAC TCT	180
Glu Tyr Thr Pro Glu Gly Lys Ser Val Lys Val Ser Arg Tyr Ser	
50 55 60	
GTA ATC CAC GGC AAG GAA TAC TTT TCC GAA GGT ACC GCC TAC CCA	225
Val Ile His Gly Lys Glu Tyr Phe Ser Glu Gly Thr Ala Tyr Pro	
65 70 75	
GTT GGT GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC TAC ACT ATT	270
Val Gly Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Tyr Thr Ile	
80 85 90	
GGA GGT GTG ACC CAG GAG GGT GTA TTC AAC GTA CTC TCC ACT GAC	315
Gly Gly Val Thr Gln Glu Gly Val Phe Asn Val Leu Ser Thr Asp	
95 100 105	
AAC AAG AAC TAC ATC ATC GGA TAC TTT TGC TCG TAC GAC GAG GAC	360
Asn Lys Asn Tyr Ile Ile Gly Tyr Phe Cys Ser Tyr Asp Glu Asp	
110 115 120	
AAG AAG GGA CAC ATG GAC TTG GTC TGG GTG CTC TCC AGA AGC ATG	405
Lys Lys Gly His Met Asp Leu Val Trp Val Leu Ser Arg Ser Met	
125 130 135	
GTC CTT ACT GGT GAA GCC AAG ACC GCT GTC GAG AAC TAC CTT ATC	450
Val Leu Thr Gly Glu Ala Lys Thr Ala Val Glu Asn Tyr Leu Ile	
140 145 150	
GGC TCC CCA GTA GTC GAC TCC CAG AAA CTG GTA TAC AGT GAC TTC	495
Gly Ser Pro Val Val Asp Ser Gln Lys Leu Val Tyr Ser Asp Phe	
155 160 165	
TCT GAA GCC GCC TGC AAG GTC AAC AAT	522
Ser Glu Ala Ala Cys Lys Val Asn Asn	
170	

INFORMATION FOR SEQ ID NO:13:

SEQUENCE CHARACTERISTICS:

LENGTH: 76 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTGGTCCCAG TACCATGGTA AATGGTGGNN KGTCGCCNNK TACCCCNKN	50
NKNNKNNKAA GTACGGAAAG TGCGGA	76

DE 199 26 068 C1

INFORMATION FOR SEQ ID NO:14:

SEQUENCE CHARACTERISTICS:

LENGTH: 1219 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: fragment of phasmid pBBP24

FEATURE:

NAME/KEY: signal peptide
LOCATION: (22..84)

FEATURE:

NAME/KEY: mature peptide
LOCATION: (85..1209)
OTHER INFORMATION:
/Product = "fusion protein composed of bilin-binding protein
Strep-tag II and fragment of phage envelope
protein pIII, with interrupted reading frame"
/Codon = (sequence: "TAG", amino acid:Gln)

FEATURE:

NAME/KEY: coding sequence
LOCATION: (85..606)
OTHER INFORMATION:
/Product = "mature bilin-binding protein with interrupted
reading frame"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (607..636)
OTHER INFORMATION:
/Product = "Strep-tag II affinity tag"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (637..639)
OTHER INFORMATION:
/Product = "amber stop codon"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (640..1209)
OTHER INFORMATION:
/Product = "amino acids 217-406 of envelope protein pIII"

SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
TCTAGATAAC GAGGGCAAAA A ATG AAA AAG ACA GCT ATC GCG ATT      45
                      Met Lys Lys Thr Ala Ile Ala Ile
                      -21 -20                      -15

GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC GAC GTG    90
Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Asp Val
          -10                      -5                      -1  1
```

DE 199 26 068 C1

TAC CAC GAC GGT GCC TGT CCC GAA GTC AAG CCA GTC GAC AAC TTC	135
Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro Val Asp Asn Phe	
5 10 15	
GAC TGG TCC CAG TAC CAT GGT AAA TGG TGG GAA GTC GCC AAA TAC	180
Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Glu Val Ala Lys Tyr	
20 25 30	
CCC AAC TCA GTT GAG AAG TAC GGA AAT TAA TGA TGG GCT GAG TAC	225
Pro Asn Ser Val Glu Lys Tyr Gly Asn Trp Ala Glu Tyr	
35 40 45	
ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG AAC TAC CAC GTA ATC	270
Thr Pro Glu Gly Lys Ser Val Lys Val Ser Asn Tyr His Val Ile	
50 55 60	
CAC GGC AAG GAA TAC TTT ATT GAA GGA ACT GCC TAC CCA GTT GGT	315
His Gly Lys Glu Tyr Phe Ile Glu Gly Thr Ala Tyr Pro Val Gly	
65 70 75	
GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC CTG ACT TAC GGA GGT	360
Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Leu Thr Tyr Gly Gly	
80 85 90	
GTC ACC AAG GAG AAC GTA TTC AAC GTA CTC TCC ACT GAC AAC AAG	405
Val Thr Lys Glu Asn Val Phe Asn Val Leu Ser Thr Asp Asn Lys	
95 100 105	
AAC TAC ATC ATC GGA TAC TAC TGC AAA TAC GAC GAG GAC AAG AAG	450
Asn Tyr Ile Ile Gly Tyr Tyr Cys Lys Tyr Asp Glu Asp Lys Lys	
110 115 120	
GGA CAC CAA GAC TTC GTC TGG GTG CTC TCC AGA AGC ATG GTC CTT	495
Gly His Gln Asp Phe Val Trp Val Leu Ser Arg Ser Met Val Leu	
125 130 135	
ACT GGT GAA GCC AAG ACC GCT GTC GAG AAC TAC CTT ATC GGC TCC	540
Thr Gly Glu Ala Lys Thr Ala Val Glu Asn Tyr Leu Ile Gly Ser	
140 145 150	
CCA GTA GTC GAC TCC CAG AAA CTG GTA TAC AGT GAC TTC TCT GAA	585
Pro Val Val Asp Ser Gln Lys Leu Val Tyr Ser Asp Phe Ser Glu	
155 160 165	
GCC GCC TGC AAG GTC AAC AAT AGC AAC TGG TCT CAC CCG CAG TTC	630
Ala Ala Cys Lys Val Asn Asn Ser Asn Trp Ser His Pro Gln Phe	
170 175 180	
GAA AAA TAG GCT GGC GGC GGC TCT GGT GGT GGT TCT GGC GGC GGC	675
Glu Lys Gln Ala Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly	
185 190 195	
TCT GAG GGT GGT GGC TCT GAG GGT GGC GGT TCT GAG GGT GGC GGC	720
Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly	
200 205 210	

DE 199 26 068 C1

```

TCT GAG GGA GGC GGT TCC GGT GGT GGC TCT GGT TCC GGT GAT TTT 765
Ser Glu Gly Gly Gly Ser Gly Gly Gly Ser Gly Ser Gly Asp Phe
      215                                220                                225

5 GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA 810
Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu
      230                                235                                240

0 AAT GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT 855
Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu
      245                                250                                255

GAT TCT GTC GCT ACT GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT 900
Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile
      260                                265                                270

GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT GGT GCT ACT GGT GAT 945
Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp
      275                                280                                285

TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT 990
Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp
      290                                295                                300

AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC 1035
Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu
      305                                310                                315

CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA 1080
Pro Gln Ser Val Glu Cys Arg Pro Phe Val Phe Gly Ala Gly Lys
      320                                325                                330

CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA AAC TTA TTC CGT 1125
Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg
      335                                340                                345

GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA 1170
Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val
      350                                355                                360

TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT          1209
Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
      365                                370                                375

TAATAAGCTT          1219

```

INFORMATION FOR SEQ ID NO:15:

SEQUENCE CHARACTERISTICS:

LENGTH: 522

TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: coding sequence of mutein DigA16

```
/Product = "mutein without fusion parts"
```

SEQUENCE DESCRIPTION: SEO ID NO:15:

GAC Asp 1	GTG Val	TAC Tyr	CAC His	GAC Asp 5	GGT Gly	GCC Ala	TGT Cys	CCC Pro	GAA Glu 10	GTC Val	AAG Lys	CCA Pro	GTC Val	GAC Asp 15	45
AAC Asn	TTC Phe	GAC Asp	TGG Trp	TCC Ser 20	CAG Gln	TAC Tyr	CAT His	GGT Gly	AAA Lys 25	TGG Trp	TGG Trp	CAG Gln	GTC Val	GCC Ala 30	90
GCG Ala	TAC Tyr	CCC Pro	GAT Asp	CAT His 35	ATT Ile	ACG Thr	AAG Lys	TAC Tyr	GGA Gly 40	AAG Lys	TGC Cys	GGA Gly	TGG Trp	GCT Ala 45	135
GAG Glu	TAC Tyr	ACT Thr	CCT Pro	GAA Glu 50	GGC Gly	AAG Lys	AGT Ser	GTC Val	AAA Lys 55	GTT Val	TCG Ser	CGC Arg	TAC Tyr	TCT Ser 60	180
GTA Val	ATC Ile	CAC His	GGC Gly	AAG Lys 65	GAA Glu	TAC Tyr	TTT Phe	TCC Ser	GAA Glu 70	GGT Gly	ACC Thr	GCC Ala	TAC Tyr	CCA Pro 75	225
GTT Val	GGT Gly	GAC Asp	TCC Ser	AAG Lys 80	ATT Ile	GGA Gly	AAG Lys	ATC Ile	TAC Tyr 85	CAC His	AGC Ser	TAC Tyr	ACT Thr	ATT Ile 90	270
GGA Gly	GGT Gly	GTG Val	ACC Thr	CAG Gln 95	GAG Glu	GGT Gly	GTA Val	TTC Phe	AAC Asn 100	GTA Val	CTC Leu	TCC Ser	ACT Thr	GAC Asp 105	315
AAC Asn	AAG Lys	AAC Asn	TAC Tyr	ATC Ile 110	ATC Ile	GGA Gly	TAC Tyr	TTT Phe	TGC Cys 115	TCG Ser	TAC Tyr	GAC Asp	GAG Glu	GAC Asp 120	360
AAG Lys	AAG Lys	GGA Gly	CAC His	ATG Met 125	GAC Asp	TTG Leu	GTC Val	TGG Trp	GTG Val 130	CTC Leu	TCC Ser	AGA Arg	AGC Ser	ATG Met 135	405
GTC Val	CTT Leu	ACT Thr	GGT Gly	GAA Glu 140	GCC Ala	AAG Lys	ACC Thr	GCT Ala	GTC Val 145	GAG Glu	AAC Asn	TAC Tyr	CTT Leu	ATC Ile 150	450
GGC Gly	TCC Ser	CCA Pro	GTA Val	GTC Val 155	GAC Asp	TCC Ser	CAG Gln	AAA Lys	CTG Leu 160	GTA Val	TAC Tyr	AGT Ser	GAC Asp	TTC Phe 165	495
TCT Ser	GAA Glu	GCC Ala	GCC Ala	TGC Cys 170	AAG Lys	GTC Val	AAC Asn	AAT Asn							522

DE 199 26 068 C1

INFORMATION FOR SEQ ID NO:16

SEQUENCE CHARACTERISTICS:

LENGTH: 1380 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: fragment of plasmid pBBP21

FEATURE:

NAME/KEY: signal peptide
LOCATION: (22..84)

FEATURE:

NAME/KEY: mature peptide
LOCATION: (85..636)
OTHER INFORMATION:
/Product = "fusion protein composed of bilin-binding protein
Strep-tag II"

FEATURE:

NAME/KEY: signal peptide
LOCATION: (658..717)

FEATURE:

NAME/KEY: mature peptide
LOCATION: (718..1365)
OTHER INFORMATION:
/Product = "DsbC protein"

SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCTAGATAAC	GAGGGCAAAA	A	ATG	AAA	AAG	ACA	GCT	ATC	GCG	ATT		45
			Met	Lys	Lys	Thr	Ala	Ile	Ala	Ile		
			-21	-20					-15			
GCA GTG GCA	CTG GCT GGT	TTC GCT	ACC GTA	GCG CAG	GCC GAC	GTG	90					
Ala Val Ala	Leu Ala Gly	Phe Ala	Thr Val	Ala Gln	Ala Asp	Val						
	-10		-5		-1	1						
TAC CAC GAC	GGT GCC TGT	CCC GAA	GTC AAG	CCA GTC	GAC AAC	TTC	135					
Tyr His Asp	Gly Ala Cys	Pro Glu	Val Lys	Pro Val	Asp Asn	Phe						
	5		10		15							
GAC TGG TCC	CAG TAC CAT	GGT AAA	TGG TGG	GAA GTC	GCC AAA	TAC	180					
Asp Trp Ser	Gln Tyr His	Gly Lys	Trp Trp	Glu Val	Ala Lys	Tyr						
	20		25		30							
CCC AAC TCA	GTT GAG AAG	TAC GGA	AAG TGC	GGA TGG	GCT GAG	TAC	225					
Pro Asn Ser	Val Glu Lys	Tyr Gly	Lys Cys	Gly Trp	Ala Glu	Tyr						
	35		40		45							
ACT CCT GAA	GGC AAG AGT	GTC AAA	GTT TCG	AAC TAC	CAC GTA	ATC	270					
Thr Pro Glu	Gly Lys Ser	Val Lys	Val Ser	Asn Tyr	His Val	Ile						
	50		55		60							

DE 199 26 068 C1

CAC	GGC	AAG	GAA	TAC	TTT	ATT	GAA	GGA	ACT	GCC	TAC	CCA	GTT	GGT	315
His	Gly	Lys	Glu	Tyr	Phe	Ile	Glu	Gly	Thr	Ala	Tyr	Pro	Val	Gly	
		65					70					75			
GAC	TCC	AAG	ATT	GGA	AAG	ATC	TAC	CAC	AGC	CTG	ACT	TAC	GGA	GGT	360
Asp	Ser	Lys	Ile	Gly	Lys	Ile	Tyr	His	Ser	Leu	Thr	Tyr	Gly	Gly	
		80					85					90			
GTC	ACC	AAG	GAG	AAC	GTA	TTC	AAC	GTA	CTC	TCC	ACT	GAC	AAC	AAG	405
Val	Thr	Lys	Glu	Asn	Val	Phe	Asn	Val	Leu	Ser	Thr	Asp	Asn	Lys	
		95					100					105			
AAC	TAC	ATC	ATC	GGA	TAC	TAC	TGC	AAA	TAC	GAC	GAG	GAC	AAG	AAG	450
Asn	Tyr	Ile	Ile	Gly	Tyr	Tyr	Cys	Lys	Tyr	Asp	Glu	Asp	Lys	Lys	
		110					115					120			
GGA	CAC	CAA	GAC	TTC	GTC	TGG	GTG	CTC	TCC	AGA	AGC	ATG	GTC	CTT	495
Gly	His	Gln	Asp	Phe	Val	Trp	Val	Leu	Ser	Arg	Ser	Met	Val	Leu	
		125					130					135			
ACT	GGT	GAA	GCC	AAG	ACC	GCT	GTC	GAG	AAC	TAC	CTT	ATC	GGC	TCC	540
Thr	Gly	Glu	Ala	Lys	Thr	Ala	Val	Glu	Asn	Tyr	Leu	Ile	Gly	Ser	
		140					145					150			
CCA	GTA	GTC	GAC	TCC	CAG	AAA	CTG	GTA	TAC	AGT	GAC	TTC	TCT	GAA	585
Pro	Val	Val	Asp	Ser	Gln	Lys	Leu	Val	Tyr	Ser	Asp	Phe	Ser	Glu	
		155					160					165			
GCC	GCC	TGC	AAG	GTC	AAC	AAT	AGC	AAC	TGG	TCT	CAC	CCG	CAG	TTC	630
Ala	Ala	Cys	Lys	Val	Asn	Asn	Ser	Asn	Trp	Ser	His	Pro	Gln	Phe	
		170					175					180			
GAA	AAA	TAATAAGCTT	CGGGAAGATT	T	ATG	AAG	AAA	GGT	TTT	ATG	675				
Glu	Lys				Met	Lys	Lys	Gly	Phe	Met					
					-20					-15					
TTG	TTT	ACT	TTG	TTA	GCG	GCG	TTT	TCA	GGC	TTT	GCT	CAG	GCT	GAT	720
Leu	Phe	Thr	Leu	Leu	Ala	Ala	Phe	Ser	Gly	Phe	Ala	Gln	Ala	Asp	
			-10						-5			-1		1	
GAC	GCG	GCA	ATT	CAA	CAA	ACG	TTA	GCC	AAA	ATG	GGC	ATC	AAA	AGC	765
Asp	Ala	Ala	Ile	Gln	Gln	Thr	Leu	Ala	Lys	Met	Gly	Ile	Lys	Ser	
			5					10					15		
AGC	GAT	ATT	CAG	CCC	GCG	CCT	GTA	GCT	GGC	ATG	AAG	ACA	GTT	CTG	810
Ser	Asp	Ile	Gln	Pro	Ala	Pro	Val	Ala	Gly	Met	Lys	Thr	Val	Leu	
			20					25					30		
ACT	AAC	AGC	GGC	GTG	TTG	TAC	ATC	ACC	GAT	GAT	GGT	AAA	CAT	ATC	855
Thr	Asn	Ser	Gly	Val	Leu	Tyr	Ile	Thr	Asp	Asp	Gly	Lys	His	Ile	
			35					40				45			
ATT	CAG	GGG	CCA	ATG	TAT	GAC	GTT	AGT	GGC	ACG	GCT	CCG	GTC	AAT	900
Ile	Gln	Gly	Pro	Met	Tyr	Asp	Val	Ser	Gly	Thr	Ala	Pro	Val	Asn	
			50					55					60		

DE 199 26 068 C1

GTC	ACC	AAT	AAG	ATG	CTG	TTA	AAG	CAG	TTG	AAT	GCG	CTT	GAA	AAA	945
Val	Thr	Asn	Lys	Met	Leu	Leu	Lys	Gln	Leu	Asn	Ala	Leu	Glu	Lys	
			65					70					75		
GAG	ATG	ATC	GTT	TAT	AAA	GCG	CCG	CAG	GAA	AAA	CAC	GTC	ATC	ACC	990
Glu	Met	Ile	Val	Tyr	Lys	Ala	Pro	Gln	Glu	Lys	His	Val	Ile	Thr	
			80					85					90		
GTG	TTT	ACT	GAT	ATT	ACC	TGT	GGT	TAC	TGC	CAC	AAA	CTG	CAT	GAG	1035
Val	Phe	Thr	Asp	Ile	Thr	Cys	Gly	Tyr	Cys	His	Lys	Leu	His	Glu	
			95					100					105		
CAA	ATG	GCA	GAC	TAC	AAC	GCG	CTG	GGG	ATC	ACC	GTG	CGT	TAT	CTT	1080
Gln	Met	Ala	Asp	Tyr	Asn	Ala	Leu	Gly	Ile	Thr	Val	Arg	Tyr	Leu	
			110					115					120		
GCT	TTC	CCG	CGC	CAG	GGG	CTG	GAC	AGC	GAT	GCA	GAG	AAA	GAA	ATG	1125
Ala	Phe	Pro	Arg	Gln	Gly	Leu	Asp	Ser	Asp	Ala	Glu	Lys	Glu	Met	
			125					130					135		
AAA	GCT	ATC	TGG	TGT	GCG	AAA	GAT	AAA	AAC	AAA	GCG	TTT	GAT	GAT	1170
Lys	Ala	Ile	Trp	Cys	Ala	Lys	Asp	Lys	Asn	Lys	Ala	Phe	Asp	Asp	
			140					145					150		
GTG	ATG	GCA	GGT	AAA	AGC	GTC	GCA	CCA	GCC	AGT	TGC	GAC	GTG	GAT	1215
Val	Met	Ala	Gly	Lys	Ser	Val	Ala	Pro	Ala	Ser	Cys	Asp	Val	Asp	
			155					160					165		
ATT	GCC	GAC	CAT	TAC	GCA	CTT	GGC	GTC	CAG	CTT	GGC	GTT	AGC	GGT	1260
Ile	Ala	Asp	His	Tyr	Ala	Leu	Gly	Val	Gln	Leu	Gly	Val	Ser	Gly	
			170					175					180		
ACT	CCG	GCA	GTT	GTG	CTG	AGC	AAT	GGC	ACA	CTT	GTT	CCG	GGT	TAC	1305
Thr	Pro	Ala	Val	Val	Leu	Ser	Asn	Gly	Thr	Leu	Val	Pro	Gly	Tyr	
			185					190					195		
CAG	CCG	CCG	AAA	GAG	ATG	AAA	GAA	TTC	CTC	GAC	GAA	CAC	CAA	AAA	1350
Gln	Pro	Pro	Lys	Glu	Met	Lys	Glu	Phe	Leu	Asp	Glu	His	Gln	Lys	
			200					205					210		
ATG	ACC	AGC	GGT	AAA	TAATTCGCGT	AGCTT									1380
Met	Thr	Ser	Gly	Lys											
			215												

INFORMATION FOR SEQ ID NO:17:

SEQUENCE CHARACTERISTICS:

LENGTH: 2009 base pairs

TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: fragment of plasmid pBBP27

FEATURE:

NAME/KEY: signal peptide

LOCATION: (23..85)

DE 199 26 068 C1

FEATURE:

NAME/KEY: mature peptide
LOCATION: (86..1999)
OTHER INFORMATION:
/Product = "fusion protein composed of alkaline phosphatase,
linker peptide Pro-Pro-Ser-Ala, mutein DigA16
and Strep tag II"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (86..1435)
OTHER INFORMATION:
/Product = "linker peptide Pro-Pro-Ser-Ala"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (1448..1969)
OTHER INFORMATION:
/Product = "DigA16 mutein"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (1970..1999)
OTHER INFORMATION:
/Product = "Strep-tag II affinity tag"

SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCTAGAACAT GGAGAAAATA AA GTG AAA CAA AGC ACT ATT GCA CTG	46
Val Lys Gln Ser Thr Ile Ala Leu	
-21 -20 -15	
GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAA GCC CGG ACA	91
Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Arg Thr	
-10 -5 -1 1	
CCA GAA ATG CCT GTT CTG GAA AAC CGG GCT GCT CAG GGC GAT ATT	136
Pro Glu Met Pro Val Leu Glu Asn Arg Ala Ala Gln Gly Asp Ile	
5 10 15	
ACT GCA CCC GGC GGT GCT CGC CGT TTA ACG GGT GAT CAG ACT GCC	181
Thr Ala Pro Gly Gly Ala Arg Arg Leu Thr Gly Asp Gln Thr Ala	
20 25 30	
GCT CTG CGT GAT TCT CTT AGC GAT AAA CCT GCA AAA AAT ATT ATT	226
Ala Leu Arg Asp Ser Leu Ser Asp Lys Pro Ala Lys Asn Ile Ile	
35 40 45	
TTG CTG ATT GGC GAT GGG ATG GGG GAC TCG GAA ATT ACT GCC GCA	271
Leu Leu Ile Gly Asp Gly Met Gly Asp Ser Glu Ile Thr Ala Ala	
50 55 60	

DE 199 26 068 C1

CGT	AAT	TAT	GCC	GAA	GGT	GCG	GGC	GGC	TTT	TTT	AAA	GGT	ATA	GAT	316
Arg	Asn	Tyr	Ala	Glu	Gly	Ala	Gly	Gly	Phe	Phe	Lys	Gly	Ile	Asp	
		65						70					75		
GCC	TTA	CCG	CTT	ACC	GGG	CAA	TAC	ACT	CAC	TAT	GCG	CTG	AAT	AAA	361
Ala	Leu	Pro	Leu	Thr	Gly	Gln	Tyr	Thr	His	Tyr	Ala	Leu	Asn	Lys	
		80						85					90		
AAA	ACC	GGC	AAA	CCG	GAC	TAC	GTC	ACC	GAC	TCG	GCT	GCA	TCA	GCA	406
Lys	Thr	Gly	Lys	Pro	Asp	Tyr	Val	Thr	Asp	Ser	Ala	Ala	Ser	Ala	
		95						100					105		
ACC	GCC	TGG	TCA	ACC	GGT	GTC	AAA	ACC	TAT	AAC	GGC	GCG	CTG	GGC	451
Thr	Ala	Trp	Ser	Thr	Gly	Val	Lys	Thr	Tyr	Asn	Gly	Ala	Leu	Gly	
		110						115					120		
GTC	GAT	ATT	CAC	GAA	AAA	GAT	CAC	CCA	ACG	ATT	CTG	GAA	ATG	GCA	496
Val	Asp	Ile	His	Glu	Lys	Asp	His	Pro	Thr	Ile	Leu	Glu	Met	Ala	
		125						130					135		
AAA	GCC	GCA	GGT	CTG	GCG	ACC	GGT	AAC	GTT	TCT	ACC	GCA	GAG	TTG	541
Lys	Ala	Ala	Gly	Leu	Ala	Thr	Gly	Asn	Val	Ser	Thr	Ala	Glu	Leu	
		140						145					150		
CAG	GAT	GCC	ACG	CCC	GCT	GCG	CTG	GTG	GCA	CAT	GTG	ACC	TCG	CGC	586
Gln	Asp	Ala	Thr	Pro	Ala	Ala	Leu	Val	Ala	His	Val	Thr	Ser	Arg	
		155						160					165		
AAA	TGC	TAC	GGT	CCG	AGC	GCG	ACC	AGT	GAA	AAA	TGT	CCG	GGT	AAC	631
Lys	Cys	Tyr	Gly	Pro	Ser	Ala	Thr	Ser	Glu	Lys	Cys	Pro	Gly	Asn	
		170						175					180		
GCT	CTG	GAA	AAA	GGC	GGA	AAA	GGA	TCG	ATT	ACC	GAA	CAG	CTG	CTT	676
Ala	Leu	Glu	Lys	Gly	Gly	Lys	Gly	Ser	Ile	Thr	Glu	Gln	Leu	Leu	
		185						190					195		
AAC	GCT	CGT	GCC	GAC	GTT	ACG	CTT	GGC	GGC	GGC	GCA	AAA	ACC	TTT	721
Asn	Ala	Arg	Ala	Asp	Val	Thr	Leu	Gly	Gly	Gly	Ala	Lys	Thr	Phe	
		200						205					210		
GCT	GAA	ACG	GCA	ACC	GCT	GGT	GAA	TGG	CAG	GGA	AAA	ACG	CTG	CGT	766
Ala	Glu	Thr	Ala	Thr	Ala	Gly	Glu	Trp	Gln	Gly	Lys	Thr	Leu	Arg	
		215						220					225		
GAA	CAG	GCA	CAG	GCG	CGT	GGT	TAT	CAG	TTG	GTG	AGC	GAT	GCT	GCC	811
Glu	Gln	Ala	Gln	Ala	Arg	Gly	Tyr	Gln	Leu	Val	Ser	Asp	Ala	Ala	
		230						235					240		
TCA	CTG	AAT	TCG	GTG	ACG	GAA	GCG	AAT	CAG	CAA	AAA	CCC	CTG	CTT	856
Ser	Leu	Asn	Ser	Val	Thr	Glu	Ala	Asn	Gln	Gln	Lys	Pro	Leu	Leu	
		245						250					255		
GGC	CTG	TTT	GCT	GAC	GGC	AAT	ATG	CCA	GTG	CGC	TGG	CTA	GGA	CCG	901
Gly	Leu	Phe	Ala	Asp	Gly	Asn	Met	Pro	Val	Arg	Trp	Leu	Gly	Pro	
		260						265					270		

DE 199 26 068 C1

AAA	GCA	ACG	TAC	CAT	GGC	AAT	ATC	GAT	AAG	CCC	GCA	GTC	ACC	TGT	946
Lys	Ala	Thr	Tyr	His	Gly	Asn	Ile	Asp	Lys	Pro	Ala	Val	Thr	Cys	
		275					280					285			
ACG	CCA	AAT	CCG	CAA	CGT	AAT	GAC	AGT	GTA	CCA	ACC	CTG	GCG	CAG	991
Thr	Pro	Asn	Pro	Gln	Arg	Asn	Asp	Ser	Val	Pro	Thr	Leu	Ala	Gln	
		290					295					300			
ATG	ACC	GAC	AAA	GCC	ATT	GAA	TTG	TTG	AGT	AAA	AAT	GAG	AAA	GGC	1036
Met	Thr	Asp	Lys	Ala	Ile	Glu	Leu	Leu	Ser	Lys	Asn	Glu	Lys	Gly	
		305					310					315			
TTT	TTC	CTG	CAA	GTT	GAA	GGT	GCG	TCA	ATC	GAT	AAA	CAG	GAT	CAT	1081
Phe	Phe	Leu	Gln	Val	Glu	Gly	Ala	Ser	Ile	Asp	Lys	Gln	Asp	His	
		320					325					330			
GCT	GCG	AAT	CCT	TGT	GGG	CAA	ATT	GGC	GAG	ACG	GTC	GAT	CTC	GAT	1126
Ala	Ala	Asn	Pro	Cys	Gly	Gln	Ile	Gly	Glu	Thr	Val	Asp	Leu	Asp	
		335					340					345			
GAA	GCC	GTA	CAA	CGG	GCG	CTG	GAA	TTC	GCT	AAA	AAG	GAG	GGT	AAC	1171
Glu	Ala	Val	Gln	Arg	Ala	Leu	Glu	Phe	Ala	Lys	Lys	Glu	Gly	Asn	
		350					355					360			
ACG	CTG	GTC	ATA	GTC	ACC	GCT	GAT	CAC	GCC	CAC	GCC	AGC	CAG	ATT	1216
Thr	Leu	Val	Ile	Val	Thr	Ala	Asp	His	Ala	His	Ala	Ser	Gln	Ile	
		365					370					375			
GTT	GCG	CCG	GAT	ACC	AAA	GCT	CCG	GGC	CTC	ACC	CAG	GCG	CTA	AAT	1261
Val	Ala	Pro	Asp	Thr	Lys	Ala	Pro	Gly	Leu	Thr	Gln	Ala	Leu	Asn	
		380					385					390			
ACC	AAA	GAT	GGC	GCA	GTG	ATG	GTG	ATG	AGT	TAC	GGG	AAC	TCC	GAA	1306
Thr	Lys	Asp	Gly	Ala	Val	Met	Val	Met	Ser	Tyr	Gly	Asn	Ser	Glu	
		395					400					405			
GAG	GAT	TCA	CAA	GAA	CAT	ACC	GGC	AGT	CAG	TTG	CGT	ATT	GCG	GCG	1351
Glu	Asp	Ser	Gln	Glu	His	Thr	Gly	Ser	Gln	Leu	Arg	Ile	Ala	Ala	
		410					415					420			
TAT	GGC	CCG	CAT	GCC	GCC	AAT	GTT	GTT	GGA	CTG	ACC	GAC	CAG	ACC	1396
Tyr	Gly	Pro	His	Ala	Ala	Asn	Val	Val	Gly	Leu	Thr	Asp	Gln	Thr	
		425					430					435			
GAT	CTC	TTC	TAC	ACC	ATG	AAA	GCC	GCT	CTG	GGG	CTG	AAA	CCG	CCT	1441
Asp	Leu	Phe	Tyr	Thr	Met	Lys	Ala	Ala	Leu	Gly	Leu	Lys	Pro	Pro	
		440					445					450			
AGC	GCT	GAC	GTG	TAC	CAC	GAC	GGT	GCC	TGT	CCC	GAA	GTC	AAG	CCA	1486
Ser	Ala	Asp	Val	Tyr	His	Asp	Gly	Ala	Cys	Pro	Glu	Val	Lys	Pro	
		455					460					465			
GTC	GAC	AAC	TTC	GAC	TGG	TCC	CAG	TAC	CAT	GGT	AAA	TGG	TGG	CAG	1531
Val	Asp	Asn	Phe	Asp	Trp	Ser	Gln	Tyr	His	Gly	Lys	Trp	Trp	Gln	
		470					475					480			

DE 199 26 068 C1

GTC	GCC	GCG	TAC	CCC	GAT	CAT	ATT	ACG	AAG	TAC	GGA	AAG	TGC	GGA	1576
Val	Ala	Ala	Tyr	Pro	Asp	His	Ile	Thr	Lys	Tyr	Gly	Lys	Cys	Gly	
		485					490					495			
TGG	GCT	GAG	TAC	ACT	CCT	GAA	GGC	AAG	AGT	GTC	AAA	GTT	TCG	CGC	1621
Trp	Ala	Glu	Tyr	Thr	Pro	Glu	Gly	Lys	Ser	Val	Lys	Val	Ser	Arg	
		500					505					510			
TAC	TCT	GTA	ATC	CAC	GGC	AAG	GAA	TAC	TTT	TCC	GAA	GGT	ACC	GCC	1666
Tyr	Ser	Val	Ile	His	Gly	Lys	Glu	Tyr	Phe	Ser	Glu	Gly	Thr	Ala	
		515					520					525			
TAC	CCA	GTT	GGT	GAC	TCC	AAG	ATT	GGA	AAG	ATC	TAC	CAC	AGC	TAC	1711
Tyr	Pro	Val	Gly	Asp	Ser	Lys	Ile	Gly	Lys	Ile	Tyr	His	Ser	Tyr	
		530					535					540			
ACT	ATT	GGA	GGT	GTG	ACC	CAG	GAG	GGT	GTA	TTC	AAC	GTA	CTC	TCC	1756
Thr	Ile	Gly	Gly	Val	Thr	Gln	Glu	Gly	Val	Phe	Asn	Val	Leu	Ser	
		545					550					555			
ACT	GAC	AAC	AAG	AAC	TAC	ATC	ATC	GGA	TAC	TTT	TGC	TCG	TAC	GAC	1801
Thr	Asp	Asn	Lys	Asn	Tyr	Ile	Ile	Gly	Tyr	Phe	Cys	Ser	Tyr	Asp	
		560					565					570			
GAG	GAC	AAG	AAG	GGA	CAC	ATG	GAC	TTG	GTC	TGG	GTG	CTC	TCC	AGA	1846
Glu	Asp	Lys	Lys	Gly	His	Met	Asp	Leu	Val	Trp	Val	Leu	Ser	Arg	
		575					580					585			
AGC	ATG	GTC	CTT	ACT	GGT	GAA	GCC	AAG	ACC	GCT	GTC	GAG	AAC	TAC	1891
Ser	Met	Val	Leu	Thr	Gly	Glu	Ala	Lys	Thr	Ala	Val	Glu	Asn	Tyr	
		590					595					600			
CTT	ATC	GGC	TCC	CCA	GTA	GTC	GAC	TCC	CAG	AAA	CTG	GTA	TAC	AGT	1936
Leu	Ile	Gly	Ser	Pro	Val	Val	Asp	Ser	Gln	Lys	Leu	Val	Tyr	Ser	
		605					610					615			
GAC	TTC	TCT	GAA	GCC	GCC	TGC	AAG	GTC	AAC	AAT	AGC	AAC	TGG	TCT	1981
Asp	Phe	Ser	Glu	Ala	Ala	Cys	Lys	Val	Asn	Asn	Ser	Asn	Trp	Ser	
		620					625					630			
CAC	CCG	CAG	TTC	GAA	AAA	TAATAAGCTT									2009
His	Pro	Gln	Phe	Glu	Lys										
		635													

INFORMATION FOR SEQ ID NO:18:

SEQUENCE CHARACTERISTICS:

LENGTH: 2005 base pairs

TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: fragment of plasmid pBBP29

FEATURE:

NAME/KEY: signal peptide

LOCATION: (22..84)

DE 199 26 068 C1

FEATURE:

NAME/KEY: mature peptide
LOCATION: (85..1998)
OTHER INFORMATION:
/Product = "fusion protein composed of DigA16 mutein, Strep tag II, linker peptide Gly(5) and alkaline phosphatase"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (85..606)
OTHER INFORMATION:
/Product = "DigA16 mutein"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (607..636)
OTHER INFORMATION:
/Product = "Strep-tag II affinity tag"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (637..651)
OTHER INFORMATION:
/Product = "linker peptide Gly-Gly-Gly-Gly-Gly"

FEATURE:

NAME/KEY: coding sequence
LOCATION: (652..1998)
OTHER INFORMATION:
/Product = "alkaline phosphatase without signal sequence and N-terminal Arg"

SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCTAGATAAC	GAGGGCAAAA	A	ATG	AAA	AAG	ACA	GCT	ATC	GCG	ATT		45
			Met	Lys	Lys	Thr	Ala	Ile	Ala	Ile		
			-21	-20					-15			
GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC GAC GTG												90
Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Asp Val												
			-10			-5			-1	1		
TAC CAC GAC GGT GCC TGT CCC GAA GTC AAG CCA GTC GAC AAC TTC												135
Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro Val Asp Asn Phe												
			5			10			15			
GAC TGG TCC CAG TAC CAT GGT AAA TGG TGG CAG GTC GCC GCG TAC												180
Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Gln Val Ala Ala Tyr												
			20			25			30			
CCC GAT CAT ATT ACG AAG TAC GGA AAG TGC GGA TGG GCT GAG TAC												225
Pro Asp His Ile Thr Lys Tyr Gly Lys Cys Gly Trp Ala Glu Tyr												
			35			40			45			
ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG CGC TAC TCT GTA ATC												270
Thr Pro Glu Gly Lys Ser Val Lys Val Ser Arg Tyr Ser Val Ile												
			50			55			60			

DE 199 26 068 C1

CAC	GGC	AAG	GAA	TAC	TTT	TCC	GAA	GGT	ACC	GCC	TAC	CCA	GTT	GGT	315
His	Gly	Lys	Glu	Tyr	Phe	Ser	Glu	Gly	Thr	Ala	Tyr	Pro	Val	Gly	
		65					70					75			
GAC	TCC	AAG	ATT	GGA	AAG	ATC	TAC	CAC	AGC	TAC	ACT	ATT	GGA	GGT	360
Asp	Ser	Lys	Ile	Gly	Lys	Ile	Tyr	His	Ser	Tyr	Thr	Ile	Gly	Gly	
		80					85					90			
GTG	ACC	CAG	GAG	GGT	GTA	TTC	AAC	GTA	CTC	TCC	ACT	GAC	AAC	AAG	405
Val	Thr	Gln	Glu	Gly	Val	Phe	Asn	Val	Leu	Ser	Thr	Asp	Asn	Lys	
		95					100					105			
AAC	TAC	ATC	ATC	GGA	TAC	TTT	TGC	TCG	TAC	GAC	GAG	GAC	AAG	AAG	450
Asn	Tyr	Ile	Ile	Gly	Tyr	Phe	Cys	Ser	Tyr	Asp	Glu	Asp	Lys	Lys	
		110					115					120			
GGA	CAC	ATG	GAC	TTG	GTC	TGG	GTG	CTC	TCC	AGA	AGC	ATG	GTC	CTT	495
Gly	His	Met	Asp	Leu	Val	Trp	Val	Leu	Ser	Arg	Ser	Met	Val	Leu	
		125					130					135			
ACT	GGT	GAA	GCC	AAG	ACC	GCT	GTC	GAG	AAC	TAC	CTT	ATC	GGC	TCC	540
Thr	Gly	Glu	Ala	Lys	Thr	Ala	Val	Glu	Asn	Tyr	Leu	Ile	Gly	Ser	
		140					145					150			
CCA	GTA	GTC	GAC	TCC	CAG	AAA	CTG	GTA	TAC	AGT	GAC	TTC	TCT	GAA	585
Pro	Val	Val	Asp	Ser	Gln	Lys	Leu	Val	Tyr	Ser	Asp	Phe	Ser	Glu	
		155					160					165			
GCC	GCC	TGC	AAG	GTC	AAC	AAT	AGC	AAC	TGG	TCT	CAC	CCG	CAG	TTC	630
Ala	Ala	Cys	Lys	Val	Asn	Asn	Ser	Asn	Trp	Ser	His	Pro	Gln	Phe	
		170					175					180			
GAA	AAA	GGT	GGC	GGC	GGT	GGT	ACA	CCA	GAA	ATG	CCT	GTT	CTG	GAA	675
Glu	Lys	Gly	Gly	Gly	Gly	Gly	Thr	Pro	Glu	Met	Pro	Val	Leu	Glu	
		185					190					195			
AAC	CGG	GCT	GCT	CAG	GGC	GAT	ATT	ACT	GCA	CCC	GGC	GGT	GCT	CGC	720
Asn	Arg	Ala	Ala	Gln	Gly	Asp	Ile	Thr	Ala	Pro	Gly	Gly	Ala	Arg	
		200					205					210			
CGT	TTA	ACG	GGT	GAT	CAG	ACT	GCC	GCT	CTG	CGT	GAT	TCT	CTT	AGC	765
Arg	Leu	Thr	Gly	Asp	Gln	Thr	Gla	Ala	Ala	Leu	Arg	Asp	Ser	Leu	
		215					220					225			
GAT	AAA	CCT	GCA	AAA	AAT	ATT	ATT	TTG	CTG	ATT	GGC	GAT	GGG	ATG	810
Asp	Lys	Pro	Ala	Lys	Asn	Ile	Ile	Leu	Leu	Ile	Gly	Asp	Gly	Met	
		230					235					240			
GGG	GAC	TCG	GAA	ATT	ACT	GCC	GCA	CGT	AAT	TAT	GCC	GAA	GGT	GCG	855
Gly	Asp	Ser	Glu	Ile	Thr	Ala	Ala	Arg	Asn	Tyr	Ala	Glu	Gly	Ala	
		245					250					255			
GGC	GGC	TTT	TTT	AAA	GGT	ATA	GAT	GCC	TTA	CCG	CTT	ACC	GGG	CAA	900
Gly	Gly	Phe	Phe	Lys	Gly	Ile	Asp	Ala	Leu	Pro	Leu	Thr	Gly	Gln	
		260					265					270			

DE 199 26 068 C1

TAC	ACT	CAC	TAT	GCG	CTG	AAT	AAA	AAA	ACC	GGC	AAA	CCG	GAC	TAC	945
Tyr	Thr	His	Tyr	Ala	Leu	Asn	Lys	Lys	Thr	Gly	Lys	Pro	Asp	Tyr	
		275					280					285			
GTC	ACC	GAC	TCG	GCT	GCA	TCA	GCA	ACC	GCC	TGG	TCA	ACC	GGT	GTC	990
Val	Thr	Asp	Ser	Ala	Ala	Ser	Ala	Thr	Ala	Trp	Ser	Thr	Gly	Val	
		290					295					300			
AAA	ACC	TAT	AAC	GGC	GCG	CTG	GGC	GTC	GAT	ATT	CAC	GAA	AAA	GAT	1035
Lys	Thr	Tyr	Asn	Gly	Ala	Leu	Gly	Val	Asp	Ile	His	Glu	Lys	Asp	
		305					310					315			
CAC	CCA	ACG	ATT	CTG	GAA	ATG	GCA	AAA	GCC	GCA	GGT	CTG	GCG	ACC	1080
His	Pro	Thr	Ile	Leu	Glu	Met	Ala	Lys	Ala	Ala	Gly	Leu	Ala	Thr	
		320					325					330			
GGT	AAC	GTT	TCT	ACC	GCA	GAG	TTG	CAG	GAT	GCC	ACG	CCC	GCT	GCG	1125
Gly	Asn	Val	Ser	Thr	Ala	Glu	Leu	Gln	Asp	Ala	Thr	Pro	Ala	Ala	
		335					340					345			
CTG	GTG	GCA	CAT	GTG	ACC	TCG	CGC	AAA	TGC	TAC	GGT	CCG	AGC	GCG	1170
Leu	Val	Ala	His	Val	Thr	Ser	Arg	Lys	Cys	Tyr	Gly	Pro	Ser	Ala	
		350					355					360			
ACC	AGT	GAA	AAA	TGT	CCG	GGT	AAC	GCT	CTG	GAA	AAA	GGC	GGA	AAA	1215
Thr	Ser	Glu	Lys	Cys	Pro	Gly	Asn	Ala	Leu	Glu	Lys	Gly	Gly	Lys	
		365					370					375			
GGA	TCG	ATT	ACC	GAA	CAG	CTG	CTT	AAC	GCT	CGT	GCC	GAC	GTT	ACG	1260
Gly	Ser	Ile	Thr	Glu	Gln	Leu	Leu	Asn	Ala	Arg	Ala	Asp	Val	Thr	
		380					385					390			
CTT	GGC	GGC	GGC	GCA	AAA	ACC	TTT	GCT	GAA	ACG	GCA	ACC	GCT	GGT	1305
Leu	Gly	Gly	Gly	Ala	Lys	Thr	Phe	Ala	Glu	Thr	Ala	Thr	Ala	Gly	
		395					400					405			
GAA	TGG	CAG	GGA	AAA	ACG	CTG	CGT	GAA	CAG	GCA	CAG	GCG	CGT	GGT	1350
Glu	Trp	Gln	Gly	Lys	Thr	Leu	Arg	Glu	Gln	Ala	Gln	Ala	Arg	Gly	
		410					415					420			
TAT	CAG	TTG	GTG	AGC	GAT	GCT	GCC	TCA	CTG	AAT	TCG	GTG	ACG	GAA	1395
Tyr	Gln	Leu	Val	Ser	Asp	Ala	Ala	Ser	Leu	Asn	Ser	Val	Thr	Glu	
		425					430					435			
GCG	AAT	CAG	CAA	AAA	CCC	CTG	CTT	GGC	CTG	TTT	GCT	GAC	GGC	AAT	1440
Ala	Asn	Gln	Gln	Lys	Pro	Leu	Leu	Gly	Leu	Phe	Ala	Asp	Gly	Asn	
		440					445					450			
ATG	CCA	GTG	CGC	TGG	CTA	GGA	CCG	AAA	GCA	ACG	TAC	CAT	GGC	AAT	1485
Met	Pro	Val	Arg	Trp	Leu	Gly	Pro	Lys	Ala	Thr	Tyr	His	Gly	Asn	
		455					460					465			
ATC	GAT	AAG	CCC	GCA	GTC	ACC	TGT	ACG	CCA	AAT	CCG	CAA	CGT	AAT	1530
Ile	Asp	Lys	Pro	Ala	Val	Thr	Cys	Thr	Pro	Asn	Pro	Gln	Arg	Asn	
		470					475					480			

[illegible]

Patent Claims

1. A polypeptide selected from muteins of the bilin-binding protein, characterized in that it
 - 5 (a) is able to bind digoxigenin or digoxigenin conjugates,
 - (b) does not bind ouabain, testosterone and 4-aminofluorescein and
 - (c) has an amino acid substitution at at least one
- 10 of the sequence positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125 and 127 of the bilin-binding protein.
2. The polypeptide as claimed in claim 1,
- 15 characterized in that the dissociation constant of the complex with digoxigenin is 100 nM or less.
3. The polypeptide as claimed in claim 1 or 2, characterized in that it carries, when compared to the
- 20 bilin-binding protein, at least one of the amino acid substitutions selected from Glu(28)->Gln, Lys(31)->Ala, Asn(34)->Asp, Ser(35)->His, Val(36)->Ile, Glu(37)->Thr, Asn(58)->Arg, His(60)->Ser, Ile(69)->Ser, Leu(88)->Tyr, Tyr(90)->Ile, Lys(95)->Gln, Asn(97)->Gly,
- 25 Tyr(114)->Phe, Lys(116)->Ser, Gln(125)->Met and Phe(127)->Leu.
4. The polypeptide as claimed in one or more of claims 1 to 3, characterized in that it carries at
- 30 least one label group, selected from enzymatic label, radioactive label, fluorescent label, chromophoric label, (bio)luminescent label or label containing haptens, biotin, metal complexes, metals or colloidal gold.
- 35
5. A fusion protein of polypeptides as claimed in one or more of claims 1 to 4, characterized in that an enzyme, another protein or a protein domain, a signal

sequence and/or an affinity peptide is fused to the amino terminus of the polypeptide in an operable manner.

5 6. A fusion protein of polypeptides as claimed in one
or more of claims 1 to 5, characterized in that an
enzyme, another protein or a protein domain, a
targeting sequence and/or an affinity peptide is fused
to the carboxy terminus of the polypeptide in an
10 operable manner.

7. A nucleic acid, characterized in that it comprises
a sequence coding for a mutein or a fusion protein of a
mutein of the bilin-binding protein as claimed in one
15 or more of claims 1 to 6.

8. A method for preparing a mutein or a fusion
protein of a mutein of the bilin-binding protein as
claimed in one or more of claims 1 to 6, characterized
20 in that the nucleic acid coding for the mutein or the
fusion protein of a mutein of the bilin-binding protein
is expressed in a bacterial or eukaryotic host cell and
the polypeptide is obtained from the cell or the
culture supernatant.

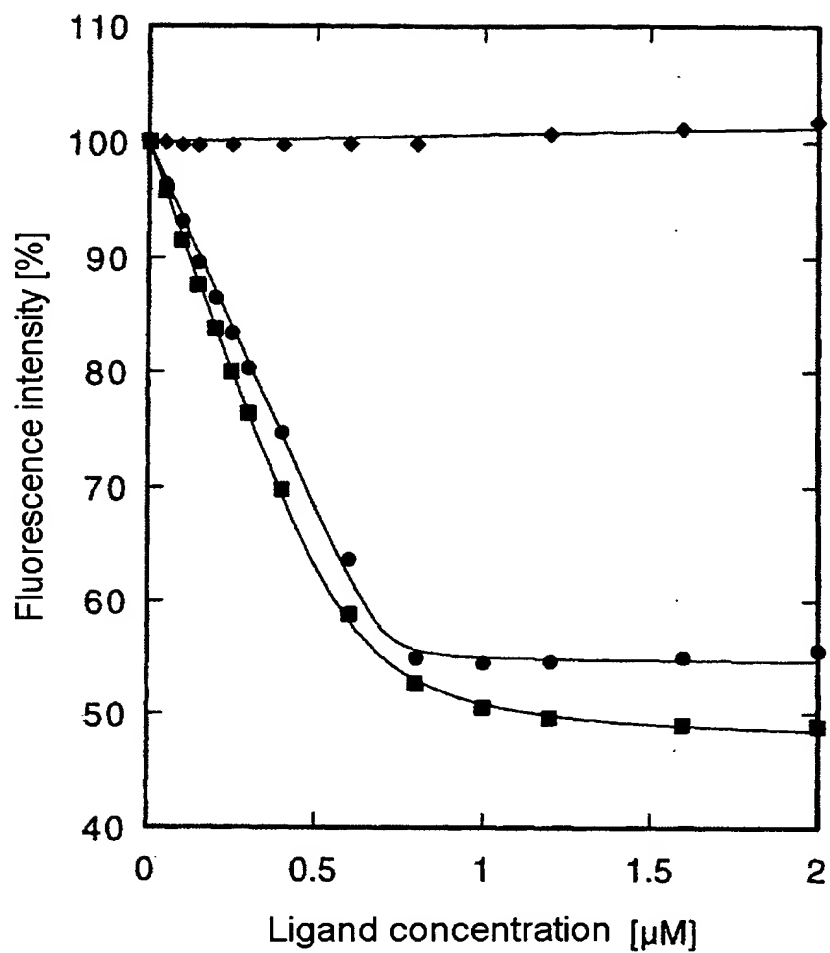
25 9. The use of a mutein or a fusion protein of a
mutein of the bilin-binding protein as claimed in one
or more of claims 1 to 8 for binding, detecting,
determining, immobilizing or removing digoxigenin or
30 conjugates of digoxigenin with proteins, nucleic acids,
carbohydrates, other biological or synthetic
macromolecules or low molecular weight chemical
compounds.

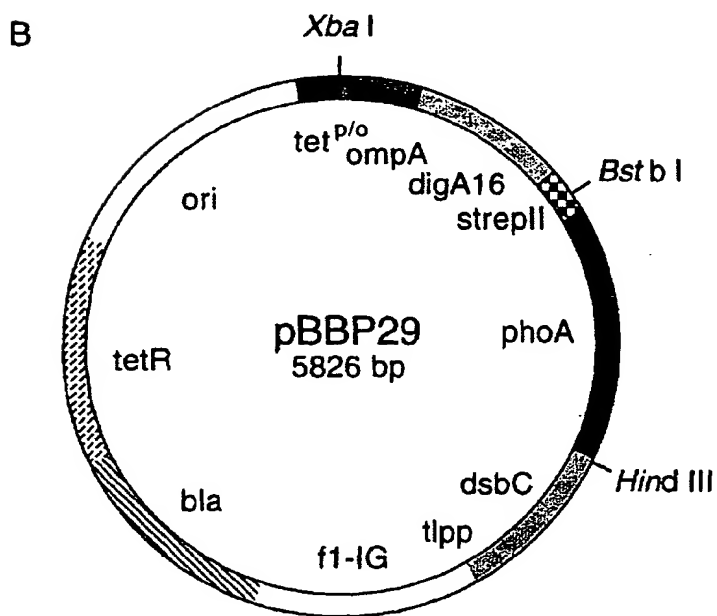
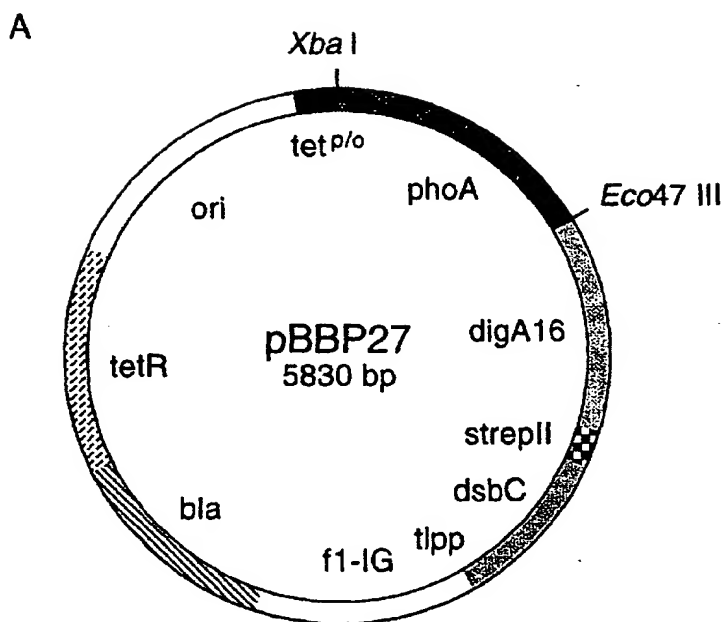
35 10. A method for detecting the digoxigenin group,
wherein a mutein of the bilin-binding protein or
a fusion protein of a mutein of the bilin-
binding protein as claimed in one or more of
claims 1 to 8 is contacted with digoxigenin or

with conjugates of digoxigenin under conditions suitable for effecting binding of the mutein to the digoxigenin group, and the mutein or the fusion protein of the mutein is determined.

5

4 pages(s) of drawings attached

**Figure 1**

**Figure 2**

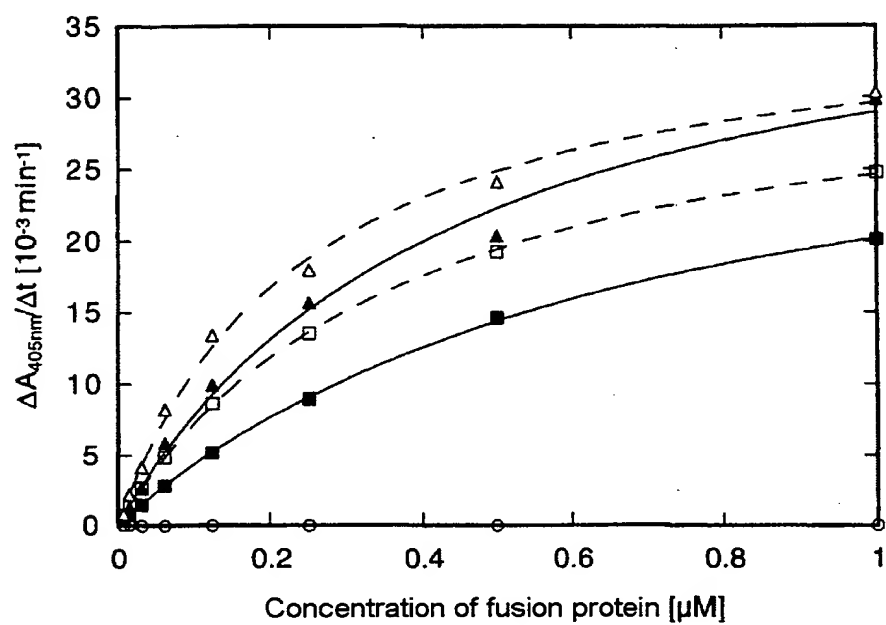


Figure 3

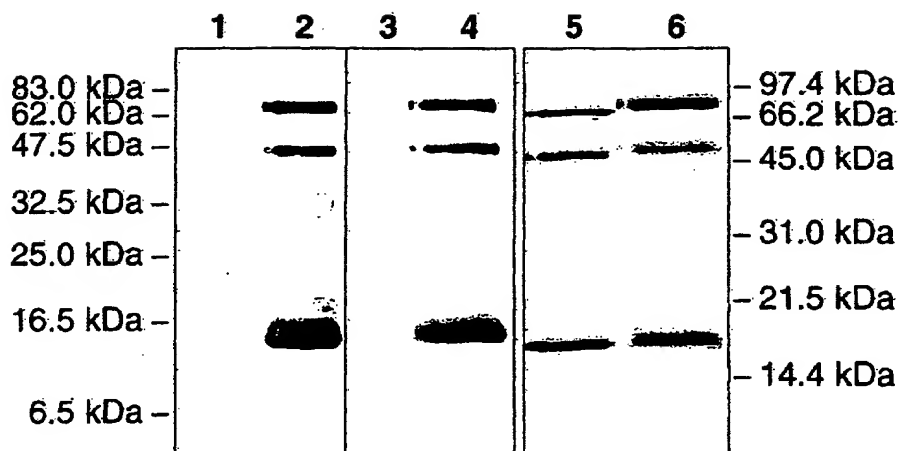


Figure 4